



# GENERAL CYTOLOGY

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## PREFACE

The present English edition of our book is not a mere translation of the original work, published in Argentina in 1946 but a complete revision. We took this opportunity to bring the text up-to-date and to add thirty four new illustrations. Many changes were made in Chapters II, IV and VI, and new material was included in Chapters V VII VIII, XI and XII. However the scope, plan and main material of the Spanish edition are kept intact here.

The book originally arose from the necessity for a synthesis in the Spanish language of the most important aspects of modern cytology.

In recent years this branch of biology has shown rapid progress and has been converted into the fundamental basis for study of the structure and function of living organisms in normal and pathological conditions. The cell can be regarded as the vital unit of organisms and the anatomical and physiological substrate of biological phenomena. In its morphological aspect modern cytology has gone beyond simple description of structures visible to the light microscope, and by the application of new methods has begun analysis of the submicroscopic organization which deals with the architectural arrangement of the molecules and micelles composing living matter. In its functional aspect, modern cytology has transcended the stage of pure description of physiological changes and seeks an explanation of these changes in the intimate physicochemical and metabolic processes of protoplasm. Finally, as a corollary, modern cytology on the basis of the physicochemical changes of the nuclear structures, has tried to interpret and explain the phenomena of heredity, sex, variation, mutation and evolution of living organisms.

In the present book an attempt has been made to stress the morphological, physiological and genetic aspects of modern cytology. Chapter I presents an introduction to the problem of the organization of living matter and a short historical summary leading to the Cell Theory and the Protoplasm Theory. Chapter II gives the chemical and physicochemical foundations of the structure and function of the cell. Chapter III describes the general microscopic organization of the cell. Chapter IV considers the submicroscopic organization or ultrastructure of protoplasm. Chapter V deals with the structure, composition and functional

significance of the cytoplasmic organoids. In Chapter VI the molecular structure of the plasma membrane and the phenomena of permeability are considered. Chapter VII describes the structure and chemical composition of the nucleus. Chapter VIII discusses the morphology and internal organization of the chromosomes and their behavior in the process of cell division, and Chapter IX correlates the chromosomes and genetic phenomena (cytogenetics).

In Chapter X the enzyme systems which take part in metabolism and respiration in the cell are described. In Chapter XI the visible manifestations of cellular activity, and in Chapter XII the phenomena of differentiation, senescence and death of the cell, are considered.

One of the most important factors stimulating the progress of cytology has been the development of new methods, in many cases derived from related sciences such as physics and biochemistry. Hence we have included brief descriptions of some of these modern techniques. The descriptions are not given as separate, isolated sections, but are closely correlated with the results obtained and with considerations of the progress in the field of cytology which these techniques have made possible.

Since many of the theories seeking to interpret cytological phenomena are still in a state of discussion, we have sought to avoid them as far as possible, and to present the reader only with established facts. Nevertheless, we have tried to point out that cytology is in a state of constant change, and that many concepts remain yet to be developed and facts to be cleared before we can establish an integrated picture of the cell from morphological and dynamic points of view.

This book is intended primarily for students of medicine, agronomy, veterinary medicine, and the biological and other natural sciences, and for those persons who, for the purposes of teaching or investigation, wish to obtain a general view of some modern aspects of cytology. With this in mind, we have included a bibliography at the end of each chapter which contains works of reference and recent papers mentioned in it.

In the preparation of the original Spanish edition we received the generous assistance of many persons. In the first place, we must express our gratitude to Dr. Manuel E. Varela for having suggested to us the writing of this book, for his unfailing interest during its preparation, and for having read a very great part of the manuscript. We wish also to express our warm thanks to Drs. V. Deulofeu, D. Brachetto-Brian, D. F. Leloir, D. Rabinovich, E. Sacerdote de Lustig, O. Nuñez and L. Primavera for critical reading and suggestions in various parts of the manuscript. We

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In connection with the present English edition we should like to express our gratitude to Drs J Bieseke, H Bunting A. R. T Denues, D E. Green, M. A. Jakus C. D Leake, A. Ormsby, C M Pomerat, F O Schmitt, J Scott and I Sizer for reading various parts of the manuscript and for many suggestions. We want particularly to thank Prof H. Stanley Bennett for his valuable suggestions and generous assistance in the preparation of all parts of the American edition. The critical comments and useful suggestions of these and other colleagues have been of great benefit to the book. Last, but not least, our thanks are due to Dr Warren Andrew for having accomplished the difficult task of translation, to Mr Donald Macdonald for his help in compiling the Index, and to the publishers, W B Saunders and Co., for their cooperation and generous acceptance of all our wishes.

EDUARDO DE ROBERTIS  
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## Chapter I

### INTRODUCTION

The cell is a fundamental morphological and physiological unit in the structure of living beings, just as is the atom in chemical structure. In addition to the organization visible with the aid of the light microscope, the cell has a further organization which, passing by micelles and organic and inorganic molecules, leads to the protons, neutrons and electrons of the atom and constitutes the ultrastructure of the cell.

If by mechanical or other means the cellular organization is destroyed, the function likewise is altered, and, although some of the vital properties may persist (for example, the activity of some cellular enzymes) the cell loses its significance as an organized unit and dies. There has ceased to exist the most important property of the living cells, autoregulation (W. Roux) in its morphological or static sense, and in its physiological or dynamic sense.

This fact gives rise to a problem which during many centuries has been fundamental in biology. Are the vital processes of a purely mechanical nature, that is, can they be interpreted on a mechanical basis (*mechanicism*) using the word mechanical in the Kantian sense, or does there exist in the organism a special force which regulates the vital properties, the structure being only a kind of frame for that which it manifests (*vitalism*)? Fortunately for modern biological investigations, this problem is of little importance and more of a metaphysical nature. The only pertinent possibility to perceive and investigate vital phenomena is that of utilizing physical and chemical methods, and all the experimental attempts that have been made to demonstrate the presence of a special agent, for example, the *entelechy* (Driesch) have failed.

The mechanist theory as well as the vitalist, resulted from the ancient tendency in biology to separate form from function. This, in part, may have been due to the preponderant influence of Aristotle, in whose *Logic* the form is a quality but function is not. We believe today that form and function constitute an

*Entelechy* from the Greek *entelecheia*, has the etymological significance to have an end. Driesch set forward the hypothesis of a principle which would regulate the unity of development and which, following Aristotle, he called "entelechy."

inseparable unity the living organism. As a consequence of this concept, a new epistemological aspect was introduced into biology that of organicism, or holism

The doctrine of organicism is based on the premise that the whole is more than the sum of its parts. In other words, the separation of a complex structure into its constituent elements does not give us an exact idea of the functioning of the structure as a whole. If for example, a watch is separated into its different parts, from the isolated observation of these parts one cannot deduce what is the function of the whole, nor that its movement is in relationship with the rotation of the earth about its axis. In spite of its appearances, the organicism, as the above cited example demonstrates, does not imply in itself anything supernatural, and the psychologists who first developed these ideas (Gestalt theory Köhler, Kafka and others) did so in accord with demonstrable facts.

The principle of holism is applicable to the inorganic world, as well as to the biological. Thus, for example, glycogen has properties very distinct from those of the molecules of glucose of which it is composed. sodium chloride is a salt which has characteristics different from the two ions which are combined in it.

In contrast to the past century which was a period of great syntheses, we are now living in an analytical period of science. We are obliged to analyze the biological processes, that is, separate them into their elements and, in order to be able to create an image of the whole, we must carry out an integration and a synthesis in the mental realm.

This holistic principle is applicable to the entire organism, but can also be applied in a very special way to the interpretation of cellular function. The cell can be considered as an organism in itself, and often very specialized, composed of many elements, the sum of which not only constitutes the cellular unit, but has a particular significance in the organism as a whole.

The Greek philosophers and naturalists, particularly Aristotle and Theophrastus, arrived at the conclusion that, "All animals and vegetables, however complicated, are constituted by few elements which are repeated in each one of them." They were referring to the macroscopic structure of the organism, such as roots, leaves and flowers common to different plants, or to the segments or organs which are repeated in the animal kingdom.

After many centuries, thanks to the invention of magnifying lenses, it was discovered that beyond the macroscopic structure there exists a whole world of microscopic dimensions, and the cell came to be regarded as "the unit of living matter" (H.

Spencer) or the primary representative of life (Claude Bernard) The single cell can constitute in itself the entire organism, as in the case of the Protozoa, or it can be grouped and differentiated into tissues and organs, as in the multicellular organisms

The development of microscopic technique permitted a more profound knowledge of cellular structure, not only as it appears in the dead cell after fixation, but likewise as it is seen in the living cell. Biochemical studies, on the other hand, demonstrated that the products of living matter and even living matter itself are constituted of the same elements which make up the inorganic world. Biochemists were able to isolate from the complex mixture which composes the cell not only fundamental substances such as proteins, fats, glycogen, nucleic acid, and so forth, but likewise other such specialized substances as hormones, vitamins and enzymes. Many of these substances can be synthesized with the methods of organic chemistry, proving that certain cellular components are able to be reproduced *in vitro*.

The advance of knowledge concerning the composition of the cell, and in particular those advances which resulted from the application of modern physical methods such as polarization optics, x ray diffraction, the ultramicroscope and the electron microscope, have caused a fundamental change in the interpretation of cellular structures.

To these discoveries one must add that of the viruses, known until recent times only by the pathological alterations which they caused in cells and by their ability to pass through filters, but which in the last few years have been observed with the electron microscope. Viruses, which undoubtedly have many properties common to the living organisms—for example, autoreproduction—are aggregates containing nucleoproteins and, in some cases, have been purified and even crystallized (Stanley) (Fig. 1).

From the historical point of view it is interesting to recall that Haeckel postulated in 1868 the existence of masses of homogeneous proteins, structureless and amorphous (Monera) as the most primitive form of organized substance. Modern studies on the viruses and our better knowledge of their properties and chemical composition confirm in part the point of view of Haeckel and appear to indicate that they might represent a bond between the inorganic and the organic world (see Pirie).

We find ourselves at present on the threshold of the era of submicroscopic biology that is to say in the era of study of the

Haeckel supposed that the "Monera" are formed directly from inorganic material.

form, aggregation and orientation of the molecules and micelles which compose the different phases of protoplasmic systems. The discovery of this submicroscopic world is of great importance, for in it, among the micelles and molecules which compose it, and the enzymes, hormones, and so forth, and the metabolites which are distributed in it, there are produced all the chemical and energy transformations which characterize vital phenomena.



Fig. 1 Electron micrograph of elementary fibrillae of tobacco mosaic virus, shadow-cast with gold.  $\times 35\,000$ . (Courtesy of R. C. Williams and R. W. G. Wyckoff.)

The modern study of living matter demonstrates that there exists a combination of levels of organization integrated among themselves, the result of which is the vital manifestation of the organism. This concept of *levels of organization*, so admirably developed by R. W. Gerard, A. E. Emerson, J. Needham, and others, permits us to refute the opinion that organization is the exclusive attribute of living beings. Needham's view is that organization exists in the entire universe, in the macro- and microcosmos, in organic material and inorganic, but that there are various levels of organization of different complexity in such a way that "The laws or rules that are encountered at one level may (in accord with the holistic principle) not appear at lower levels." The modern physicists, chemists and biologists have commenced to construct bridges between these different levels in

organization, but the road is arduous and the majority of problems continue open for future investigations, mathematical treatment and philosophical speculation

## HISTORY OF CYTOLOGY

### Discovery of the Cell Cell Theory

The discovery of the cellular structure of organisms is intimately bound up with the invention of the compound microscope. (Microscope Gr *Mikros* small and *Skopein* to see, to examine.)

The origin and the successive improvements of the microscope are difficult to trace with certainty but it is certain that its invention, of such great importance to the progress of biology can be attributed to the influence of the industry of spectacle making upon optical science. Some of the optical properties of curved surfaces were recognized by Euclid (590 B.C.) Ptolemy (127-151 A.D.) and by Alhazan at the beginning of the eleventh century; but practical use had not been made of the quality of magnification by such curved surfaces. Spectacles were invented in Italy about 1285 by Salvino de Arucati. In the sixteenth century Leonardo da Vinci and Maurolyco stressed the advantages of the use of lenses for the study of small objects. Meanwhile, a Dutch maker of spectacles in Middleburg, Zachary Janssen, and his brother Francis, making use of the experience of their father a famous optical worker discovered in 1590 how to combine two convex lenses in the interior of a tube, thus obtaining an optical instrument for magnifying minute objects. The compound microscope was thus discovered. Some historians say that, among others, the following also contributed to this invention Leonard and Thomas Diggs, De la Porta, and Mouffit. In the book "Magna Naturalis," published in 1588, Porta establishes the principles of crystalline lenses.

Galileo in 1610 combined the lenses in a tube of lead, basing his work on the rumor that a Dutchman (Janssen) had constructed such an instrument, inventing thus his microscope. The makers of spectacles improved the techniques for the polishing of lenses of short focus until in 1644 Leeuwenhoek, a humble Dutch merchant, was able to polish lenses of a magnification satisfactory for his discovery of certain "very little animalcules."

Descartes (1637) in his book "Dioptrique," describes a compound microscope composed of two lenses, one (ocular) planoconcave and the other (objective) biconvex.

Roger Bacon esteemed the value of lenses as an aid to vision but after being imprisoned, his works were unknown until 1733 so that his contributions to optics unfortunately could not have influence on the development of the microscope.

Borel, a physician in the court of Louis XIV was among the first to employ the microscope. Faber of Bamberg, a physician resident in Rome in the service of Pope Urban VII, gave the name of "microscope" to this optical instrument. Faber was a member of the Academy of Dei Lincei, to which belonged Stelluti, who in 1625 first applied the microscope to anatomical studies.

Athanasius Kircher in the seventeenth century was the first to employ the microscope systematically in the study of diseases, although he erred in his interpretations. His microscope, like the earlier models, consisted of combined lenses in the interior of a tube. This tube could be placed horizontally and brought close to the object by a simple screw furthermore, it was provided with a condenser interposed between the light source and the preparation. Robert Hooke modified the optical instrument of Kircher. Monconys and Campani (1665) intercalated a third lens between the objective and the ocular with the purpose of eliminating chromatic aberration.

Along with the mechanical development of the microscope, observations were progressing on different objects examined with it.

In 1665 Robert Hooke presented before the Royal Society of London the results of his investigations on "The texture of cork by means of magnifying lenses," and this was the point of departure of all knowledge concerning the microscopic organization of living matter. It is interesting to follow in his description this genial man of science who was at the same time a physicist and mathematician, besides being one of the best mechanics and inventors of his age.

Hooke wrote, in his "Micrographia" (Observ. XVIII. Of the Schematism or Texture of Cork, and of the Cells and Pores of some other such frothy bodies): I Took a good clear piece of Cork, and with a Pen knife sharpened as keen as a Razor I cut a piece of it off, and thereby left the surface of it exceeding smooth, then examining it very diligently with a Microscope, me thought I could perceive it to appear a little porous; but I could not so plainly distinguish them, as to be sure that they were pores, much less what Figure they were of: But judging from the lightness and yielding quality of the Cork, that certainly the texture could not be so curious, but that possibly if I could use some further diligence, I might find it to be discernable with a Microscope, I with the same sharp Pen knife, cut off from the former smooth surface an exceeding thin piece of it, and placing it on a black object Plate, because it was it self a white body and casting the light on it with a deep plano convex Glass, I could exceeding plainly perceive it to be all perforated and porous, much like a Honey-comb, but that the pores of it were not regular yet it was not unlike a Honey-comb in these particulars

First, in that it had a very little solid substance, in comparison of the empty cavity that was contained between, as does more manifestly appear by the Figure A and B for the Interstitia, or walls (as I may so call them) or partitions of those pores were neer as thin in proportion to their pores, as those thin films of Wax in a Honeycomb (which enclose and constitute the sexangular cells) are to theirs

Next, in that these pores, or cells were not very deep, but consisted of a great many little Boxes, separated out of one continued long pore, by certain Diaphragms

I no sooner discerned these (which were indeed the first microscopical pores I ever saw and perhaps, that were ever seen, for I had not met with any Writer or Person, that had made any mention of them before this) but me thought I had with the discovery of them, presently hunted to me the true and intelligible reason of all the Phenomena of Cork; As,

First, if I enquir'd why it was so exceedingly light a body? my Microscope could presently inform me that here was the same reason evident that there is found for the lightness of froth, an empty Honey-comb, Wool, a Sponge, a Pumicestone, or the like, namely a very small quantity of a solid body extended into exceeding large dimensions.

As we see, he thus left to us the name of cell (Gr *Kytos* hollow space) †

Hooke, Robert *Micrographia* (1665) Facsimile Edition published by R. T. Gunther in "Early Science in Oxford," Vol. XIII The Life and Works of Robert Hooke (Part V) Oxford, 1938, pp 112-113

† It is interesting to note that Roger Bacon, according to D'Arcy Thompson, already held a correct notion of the cell theory calling the elements, "cells"

Later and also in the seventeenth century, Grew and Malpighi repeated the observations of Hooke in different plants and recognized in them minute cavities in the midst of a homogeneous mass, which they called 'utricles or vesicles. This knowledge remained almost stationary up to the beginning of the nineteenth century. By that time there had been recognized only one of the parts elaborated by cells, the cellulose membrane of plants which constituted the cell of Hooke, or the utricle of Grew and Malpighi. The true cell contained in these cavities continued to escape the observation of the investigators.

Although the theory that all animal and plant organisms are composed of cells is associated with the names of Schleiden (1838) and Schwann (1839) numerous investigators had previously promulgated this same theory in a form more or less complete.

Thus Mirbel (1808-09) arrived at the conclusion that plants are formed by a membranous cellular tissue. Likewise the celebrated naturalist Lamarck (1809) affirmed no body can have life if its constituent parts are not cellular tissue or are not formed by cellular tissue. Similar ideas are encountered in the publications of Dutrochet (1824) Turpin (1826) Meyen (1830) von Mohl (1831) in which the cell theory is clearly expressed (See Conklin, 1939).

In spite of all these antecedents, one of the most surprising facts in the history of science is that in many texts of biology the writers consider Schleiden (1838) Professor of Botany at Jena, founder of the cell theory as though he had discovered for the first time that all tissues of plants are composed of cells (Conklin). The results of Schleiden upon the constitution of living matter in plants were confirmed and extended to animals by Schwann (1839). This author carried out a minute investigation of the tissues of the animal body and of the development of cells and for the first time used the term Cell Theory for the concept that "The cells are organisms and animals as well as plants are aggregates of these organisms, arranged in accordance with definite laws. The results of Schwann permitted the establishment in definite form of the cell theory.

It is interesting to recall in this regard that Schwann conceived very definite ideas not only on the morphological importance of the cell but likewise on its physiological significance. According to him, cellular phenomena could be divided into two groups "*the plastic phenomena*," that is, the combination of molecules which form the cell, which, in modern terms, corresponds to the cellular morphology and "*the physiological phenomena*" that result from "chemical changes, whether in the particles that compose the cell itself, or in the surrounding cytoplasm (the protoplasm of today)." These processes he designated "*metabolic phenomena*." With these words, written 100 years ago, Schwann expressed our present point of view and for this reason he may be considered as the father of modern cytology.

Owing to the eminently morphological tendency which held sway in the nineteenth century biologists dedicated their studies to the first group of phenomena according to the classification of Schwann, completely forgetting that, besides its structure, the cell likewise has physiological processes. It is also interesting to recall that the words "metabolic" and "metabolism," conceived by this anatomist, were taken up by physiology.

The cell theory was quickly extended to unicellular organisms as it was recognized that Protozoa are animals consisting of a single cell (von Siebold, 1845) and it was Haeckel who divided the animal kingdom into its two most important groups Protozoa and Metazoa.

Albert Kölliker, the famous Swiss anatomist, applied the cell theory to embryology. In 1841 he demonstrated that spermatozoa are histological elements which originate in the organism, and in 1844 he extended this concept to the ovum from which—by division of cells—the organism is developed.

Another important adaptation of the cell theory came from a completely different field. R. Virchow applied this theory to pathology demonstrating that pathological processes take place in the cells and tissues (1858).

### Protoplasm Theory

During the seventeenth and eighteenth centuries only the membrane of the plant cell had been recognized, at the beginning of the nineteenth century the attention of the investigators became concentrated on the content of the cell, which was described by different authors as 'gelatinous juice,' or 'mucous, mucilaginous.' In the cellular juice of cells of the orchid, Brown (1831) discovered the nucleus, one of the fundamental and constant components of the cell.

In 1835 Dujardin carried out observations in vivo on lower organisms such as the Rhizopoda and Foraminifera and described the content of the cell, which he called *sarcodé*, as 'a gelatinous substance, perfectly homogeneous, elastic, contractile, diaphanous, insoluble in water and without traces of organization. This substance, the organoleptic and physicochemical properties of which were described in such a precise form by Dujardin, received from Purkinje and von Mohl the name of *protoplasm* \* which has persisted up to our time.

Max Schultze, in 1861 established the essential similarity which exists between *sarcodé* and *protoplasm* of animal and plant cells, thus stating the theory which later was called the 'Protoplasm Theory' (O Hertwig, 1892).

This theory the concept of which is more general than the Protoplasm. Gr. *Prótos* first and *Plasma*, formation.

cell theory states that the cell is an accumulation of living substance or protoplasm, definitely limited in space and possessing a nucleus and a cell membrane. There are, however, organisms in which cell structure is not well manifested but which, nevertheless, are constituted by protoplasm such as bacteria and plasmodia (See Chapter VII)

The primitive concept of the cell has been converted into that of a delimited mass of protoplasm (cytoplasm) which surrounds the nucleus. For this unit the name "protoplast" (Hanstein, 1880) is more appropriate than that of "cell," although this latter has persisted.

Once these fundamental theories were established, the progress of histological knowledge was extremely rapid. The extraordinary modifications that are produced in the nucleus at each cell division attracted the attention of a great number of investigators. Thus there was discovered the phenomenon of *amitosis* or direct division (Remak, 1841) and that of indirect division (Schneider Strasburger) or *karyokinesis* (Schleicher 1879) likewise called *mitosis* (Flemming). It was proven that the fundamental fact in mitosis is the formation of the nuclear filaments or chromosomes (Waldeyer 1890) and their equal division between the nuclei or daughter cells (Flemming Strasburger van Beneden, Rabl). Another discovery of cardinal importance was that of fertilization or fecundation of the ovum and the fusion of the two pronuclei (O Hertwig 1875).

In the cytoplasm there were discovered the cell center (van Beneden, Boveri) the chondriome (Altmann, Benda) and the reticular apparatus (Golgi).

Simultaneously with the study of the tissues or cellular aggregates, investigators were increasing their concentration on the cell considered as a fundamental unit. In 1892 O Hertwig published his monograph called '*The cell and the tissues*' in which, basing his views on the characteristics of the cell, its structure and function, he made a general synthesis of biological phenomena. He demonstrated in this book that the solution of biological problems would be found in cellular processes. In this way he formed a separate branch of biology that of *cytology*, the purpose of which is, at present, the morphological, chemical and functional study of the cell.

The history of the development of cytological knowledge from the beginning of the twentieth century up to our time is found scattered throughout the chapters of this work, which is designed particularly to bring out the modern aspects and the present day orientation of cytology.

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## *Chapter II*

# CHEMICAL AND PHYSICOCHEMICAL ORGANIZATION OF THE CELL

Purely morphological cytology based on the use of the classical methods of fixation and staining has approached a static state, having exhausted the study and the description of various cellular structures. Modern cytology essentially dynamic, utilizes some of the methods of physics, chemistry and biochemistry and applies many of their results to interpret the nature of the intracellular processes and functional significance of cellular structures. For these reasons, it is convenient to begin this study with a short review of the chemical structure of the cell. At the same time, a general summary of some of the important properties of these components will be mentioned in order to give the reader a basis for understanding the following chapters. This summary is, of course, in no way complete nor does it pretend to be so. Emphasis will be placed on chemical composition from the point of view of the configuration and arrangement of the molecules constituting the structure of the cell. (See Chapter IV)

## GENERAL CHEMICAL COMPOSITION

Various types of cells and tissues show chemical differences comparable to their morphological variations. Thus, for example, the composition and metabolism of an epithelial cell of the thyroid gland differs markedly from that of muscle fibers. However there are certain substances generally found in all tissues. These may be classified as inorganic (water and mineral ions) and organic (proteins, carbohydrates, lipids, and so forth). Certain complex organic compounds with specific activities, such as hormones, vitamins and enzymes, will merely be mentioned in this chapter. They will be studied from the cytological point of view in subsequent parts of this book, particularly in Chapter X.

An early approach used by biochemists seeking to learn more of the composition of the body was to make an analysis of whole carcasses or of whole organs or tissues such as liver or brain or skin. Such an approach has only limited cytological value, as the material analyzed was ordinarily composed of a mixture of cell types. For example, skin as analyzed by biochemists usually con-

tains both epidermis and the connective tissue, dermis or corium. Overall analysis of this complex structure may be difficult to interpret in terms of specific components or changes in any one of the diverse cell types included in the sample. Nevertheless, this approach was useful as a starting point.

Table I gives an approximate idea of the relative amounts of principal inorganic and organic compounds found in active protoplasm. At the same time a rough estimate of the relative number of molecules is determined by using definite percentages and average molecular weights for the different compounds.

TABLE I

RELATIVE COMPOSITION AND RELATIVE NUMBER OF MOLECULES IN PROTOPLASM MATERIAL  
(From Sponsler and Bath, 1942.)

Substance	Percentage of Fresh Weight	Percentage Used	Average Molecular Weight Used	Approximate Relative Number of Molecules
Water	85-90	85	18	18,000
Protein	7-10	10	36,000	1
Fatty Substances	1-2	2	700	10
Other Organic Substances	1-1.5	1.5	250	60
Inorganic Material	1-1.5	1.5	53	100

Although these figures are only approximate they give an interesting picture of the relative molecular population which might exist in protoplasm.

## Water

Analysis of tissues shows that, with some exceptions, water is the component which occurs in greatest amounts. Water serves as a natural solvent for mineral ions and other substances and as a medium for dispersion which is extremely important for the colloidal structure of the protoplasm. Furthermore, it is indispensable in metabolic processes since enzymatic activity takes place exclusively in the presence of water.

The quantity of water varies a great deal from one tissue to another. Thus, for example, dentine contains only 10 per cent of water while skeletal muscle contains about 75 per cent. Even in a single organ, its different parts may present differences in water content such as the case with the brain in which the white substance contains 68 per cent, whereas the gray matter contains

84 per cent. There is some relationship between water content and the metabolic activity of the tissue. Thus the water content and the oxygen consumption both increase in the following series of tissues: fatty tissue, bone, tendon, myelin, muscle, thyroid, and gray matter. The quantity of water in the cells varies also with age: in older individuals there is less water in the tissues than in younger. The water content of adult brain is about 78 per cent and of new born rat brain about 90 per cent. Embryonic tissues are, in general, much richer in water than adult tissues. The water content of dormant tissue may be reduced to about 30 per cent of the total weight.

Water exists in free and bound form within the organism. Free water is the chief solvent in the cell and a medium for metabolic processes. Bound water is mainly tied to the polar groups of proteins by hydrogen bonds.\* As will be mentioned later, the long polypeptide chains of the protein molecules (Fig. 27) have numerous side chains, some of which possess hydrophilic groups such as  $-\text{OH}$ ,  $-\text{COOH}$ ,  $=\text{O}$ ,  $-\text{NH}_2$ ,  $-\text{NH}$ ,  $=\text{N}-$ ,  $-\text{SH}$ . Such groups can bind water molecules. The amide groups of the polypeptide chain itself also may bind water to a lesser extent. It has been calculated that in gelatin each amino acid is capable of binding 2.6 molecules of water (Sponsler). It is very difficult to calculate the amount of water which is bound by a tissue. Any resulting figure depends a great deal on the technique employed and the concept used in defining the meaning of bound water. However, as Bull mentions, if proteins constitute about 10 per cent and water about 80 per cent of the total weight of the tissue and if one gram of dry protein binds 0.35 grams of water, it can be calculated that 4.5 per cent of the total water is bound. (For more details on this problem see Gortner-Bull.)

The problem of bound water will also be discussed later in this chapter in connection with the hydrophilic colloids and the process of coacervation.

## ORGANIC COMPONENTS OF THE CELL

### Proteins and Amino Acids

Proteins are the most important component in the molecular organization of cells and are indispensable in the maintenance of vital processes. Cell organoids and enzymes also have important protein components. Long protein molecules such as

Hydrogen bonds are essentially electrostatic bonds in which a hydrogen atom is bound by two electronegative atoms (i.e.  $\text{O}-\text{H}-\text{N}$ ). Thus the hydrogen of the water molecules can form a kind of bridge between the two atoms.

collagen and fibrinogen may be found in intercellular spaces and may likewise play an important role in the organization of tissue. (See Chapter IV)

The proteins may be classified as simple, conjugated and derived. *Simple proteins* are those which, on hydrolysis, yield exclusively amino acids. In this group the most important are the *albumins* (soluble in water and coagulable by heat) the *globulins* (insoluble in water, but soluble in acids, alkaline and salt solutions), the *histones* (soluble in water insoluble in dilute ammonia, and with a strong alkaline reaction) some of which can combine with nucleic acids to form nucleohistones, which are found in large amounts in many cells (thymus, pancreas), and the *protamines* (soluble in water and incoagulable by heat, also with a strong alkaline reaction) to which belong proteins found in the spermatozoa of various fishes (clupeine, salmine, and so on) Like the histones, the protamines can also combine with nucleic acid to form nucleoproteins

*Conjugated proteins* are those in which a simple protein is combined with another substance, called the prosthetic group. To these compounds belong the *nucleoproteins*, which play an important role in the cell and whose prosthetic group is the nucleic acid, the *glycoproteins* (*mucoproteins*) in which protein is combined with carbohydrate (mucin, and so on) the *phosphoproteins*, combined with phosphorus (casein and vitellin) the *lecithoproteins*, such as fibrinogen of the blood, whose prosthetic group is lecithin and the *chromoproteins*, a very widely distributed group which includes a series of substances of great biological importance which are characterized by their particular colors. To the *chromoproteins* belong hemoglobin, in which the globin is combined with an iron porphyrin compound (heme) and the *hemocyanins* which occur in the blood of various invertebrates and in which copper is found occupying a position similar to that of the iron in hemoglobin. A series of *respiratory enzymes* (cytochromes, flavoproteins, etc.) also belong to this group

*Derived proteins* include denatured (coagulated) proteins, as well as partly hydrolyzed proteins. This group includes proteoses, peptones and polypeptides

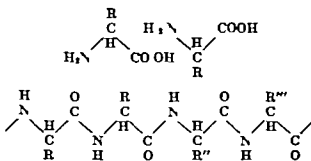
All the proteins are composed of amino acids, which are derived from aliphatic acids (like acetic acid) by replacement of an  $\alpha$  hydrogen by the amino group,  $\text{NH}_2$ , in the  $\alpha$ -position (hence their name  $\alpha$ -amino acids). The amino acids can be obtained from the proteins by hydrolysis, by enzymes, or by boiling with strong acids and bases

Table II shows the amino acid content of certain proteins found in animal tissues

TABLE II  
THE AMINO ACID CONTENT OF SOME PROTEINS  
From Sahyun, Amino Acids and Proteins  
(Reinhold Publishing Corp.)

Amino Acids (Percentage)	Casein	Egg Albumin	Fibrin (Cattle)	Cel- lin	Hemo- globin	Kera- tin (Wool)	Pep- sin	Sal- mine	Serum Al- bumin	Serum Glob- ulin
Alanine	1.9	2.2		5	4.2	4.4			2.7	2.2
Asparagine	2.3	1.2		0.4	1.2	1.4	1.4		1.3	
Arginine	3.8	5.6	7.7	8	3.6	10.2	1.3	8-4	4.9	5.2
Aspartic Acid	6.0	8.1	3.0	3.4	8.9	7.3	6.8		3.1	2.5
Cystine	0.4	1.8	1.5	0.2	1.0	13.1	2.2		5.7	1.1
Glutamic Acid	21.8	16.1	14.1	3.5	6.3	13.0	18.6		1.6	8.2
Glycine	0.3	0		23.3		0.6			0	3.3
Histidine	2.3	1.3	2.3	2.0	6	0.7	0.3		3.4	0.9
Hydroxyproline	0.2			14						
Isoleucine and Leucine	9	10.7		7.1	29.0	11.3			20.0	18
Lysine	6.3	5.1	10.1	3.0	8.1	2.8	1.7		13.2	6.2
Methionine	3.3	2.4	2.6			0				
Phenylalanine	3.9	5.1		1.4	4.2				3.1	3.8
Proline	8.7	4.2	5.1	17.3	2.3	4.4		11.0	1.0	2.8
Serine	3.8			3.3	1.0	2.9		7.8	0.6	
Threonine	4.0			1.4						
Tryptophane	1.2	1.3	3.0	0	1.3	1.8	2.2		0.3	2.3
Tyrosine	6.6	4.2	6.3	0	3.2	4.8	10.3		4.8	6
Valine	9	2.3		0		2.8		4.3		

One of the most important characteristics of the amino acids is their capacity of combining with each other to form long chains. This property is due to the presence of a carboxyl group ( $-\text{COOH}$ ) and a basic amino group ( $-\text{NH}_2$ ) in each molecule. Such substances, which contain at the same time acid and basic groups, are called *amphoteric*. The condensation of amino acids occurs in such a way that the acid group of one molecule combines with the basic group of another molecule with the loss of one molecule of water.



In this equation R, R', etc., represent a different group or residue for each amino acid. The linkage  $R-NH-CO-R$  is familiar to organic chemists as the amide linkage. In proteins this bonding is often called a peptide linkage or bond, and whole protein molecules can be regarded as high polymers belonging to the larger class known as polyamides, of which nylon is a familiar example. The new compound formed by the above reaction preserves the amphoteric character of amino acids, since it possesses at one end an acid (right) and on the other a basic group (left). This makes possible further condensations with more amino acids, forming long chains called peptides (H. Fischer). A peptide composed of only two amino acids is a dipeptide, when there are several amino acid residues the compound is known as polypeptide. In Fig. 27 a model of a polypeptide chain is shown in which the central zigzag backbone portion and the various amino acid residues extending at right angles on both sides are represented. The distance between two peptide links is about 3.5 Å. This corresponds to a single amino acid residue. If we consider the case of a protein with a molecular weight of 36,000 and of about 300 amino acid residues, the chain, when fully extended, will have a length of about 1,000 Å, a width of about 10 Å due to the presence of the amino acid residues, and a thickness of about 4.5 Å. (Sponsler and Bath.)

However, much heavier protein molecules are found in nature, some of which are listed herewith:

Insulin	36,500
Trypsin	35,500
Pepsin	43,000
Egg albumin	63,000
Hemoglobin (man)	66,700
Thyroglobulin (pig)	630,000
Rabbit papilloma virus	47,100,000

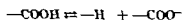
Although proteins are generally described as consisting of long polypeptide chains, the actual molecular shape of the molecules may vary considerably. On the basis of the results obtained with such methods as the use of viscosity-concentration curves, ultracentrifugation, double refraction of flow x-ray analysis, diffusion, electron microscopy and so on, two main types of proteins are often described: (1) the fibrous proteins and (2) the corpuscular or globular proteins. Among fibrous proteins, keratin, myosin, actin (Fig. 138) and collagen (Fig. 29) have biological interest. Globular proteins include the truly crystalline proteins,

such as egg albumin, serum albumin, hemoglobin, hemocyanin, pepsin, trypsin, and other crystalline enzymes.

In Chapter IV the shape of the protein molecules in relation to its molecular organization and its special relationship with other protein molecules will be considered in more detail. Here it will be merely stated that globular protein molecules are thought of as approaching a sphere in shape, whereas fibrous proteins may consist of greatly elongated elements. The actual configuration of bonds and atoms constituting these bodies is under dispute. Some believe they are made up of folded polypeptide chains (Astbury) whereas Wrinch postulates a cage-like cyclol structure. Those who adhere to a folded chain structure have postulated various arrangements of chains involving different degrees of folding (Fig 28) ( $\alpha$   $\beta$  and  $\gamma$  or supercontracted state of Astbury)

We have seen so far that the presence of a continuous peptide chain is regarded as characteristic of all proteins. It is then assumed that differences found in proteins belonging to different species or tissues or even to a single cell type may depend in part on the amino acid residues (Table II). Of the numerous residues found in cellular proteins some of them, like those belonging to leucine and phenylalanine, are aliphatic and nonpolar so that they have no affinity for water. On the contrary other residues containing  $-\text{OH}$ ,  $-\text{COOH}$  or  $-\text{NH}_2$  groups are polar and can coordinate water molecules, mainly by hydrogen bonds. Particularly interesting are the amino acid residues having groups which may become ionized and thus bear an electrical charge. Among these one finds two general types

1 Acidic groups, which may lose a proton and become negatively charged. This type is found in the diacidic amino acids like aspartic and glutamic acid, in which the free carboxyl group dissociates as follows



2 Basic groups, which by gaining a proton become positively charged. This type is found in the amino acids with two basic groups such as lysine, arginine and histidine, in which the free amino group, the guanidine group and the imidazolic group may become ionized and have a positive charge. All these so-called *ionogenic groups* together with the terminal free carboxyl and amino groups contribute to the acid base reactions of proteins and to the electrical properties of the protein molecules.

Because of the presence of these ionogenic groups, proteins like the free amino acid show an amphoteric character and form *zwitterions*. As the dissociation of the different acidic and basic groups takes place at different H<sup>+</sup> concentrations of the medium, this greatly influences the total charge of the molecule. In each type of protein there is a definite pH at which the positive and negative charges are zero. This particular pH is called the *isoelectric point* (pI). At the isoelectric point proteins in an electrical field do not migrate to either of the poles, while at lower pH they migrate to the cathode and at higher pH to the anode, this movement being called *electrophoresis*. At the isoelectric point many of the physicochemical properties of the proteins are unique. For instance, the viscosity, solubility, isoelectric osmotic pressure and conductivity reach their minimum.

The isoelectric point is characteristic for each kind of protein and depends on the type and amount of ionogenic groups contained in the molecule. For example, in the case of the histones and protamines found mainly in the nucleus, the isoelectric point is at high pH (10 to 12). This is due to the presence of numerous basic residues. In gelatin the isoelectric point is at pH 4.7.

The acid-base binding capacity resulting from the presence of positive and negative charges in the proteins and in other compounds can be studied cytologically by staining sections with acid or basic dyes in appropriate ranges of pH and under different conditions. This method has been used to determine the isoelectric point of a part of the cell (Pischniger) and to characterize different compounds present in cells and tissues (Dempey and Singer).

### Carbohydrates

The carbohydrates are composed of carbon, hydrogen and oxygen. They are important sources of energy for animal and plant cells and in many plants they are important constituents of cell walls and serve as supporting elements for the growth of plants. Plants possess the capacity of synthesizing a variety of carbohydrates directly from carbon dioxide and water in the presence of light. In animal tissues there are fewer carbohydrates, the most important being glucose, galactose, glycogen and amino sugars and their polymers.

The most important carbohydrates, from the biological point of view, are divided into four classes: monosaccharides, disaccharides, trisaccharides and polysaccharides. The first three are grouped under the common name of sugars (sac-

of their sweet taste) are readily soluble in water and alcohol, crystallize and easily pass across dialyzing membranes. The polysaccharides, on the other hand, form colloidal solutions with water do not crystallize and do not traverse membranes.

The *monosaccharides* are simple sugars with an empirical formula  $C_n(H_2O)_n$  and are classified, in accordance with the number of carbon atoms, as trioses, pentoses, hexoses and heptoses. It has been possible to synthesize more complex monosaccharides with 8 to 10 atoms of carbon such compounds, however do not seem to exist in nature. Of the monosaccharides the most important in cells are the pentoses and hexoses. Among the pentoses, ribose and desoxyribose intervene, as it will be seen later in the constitution of nucleic acids and nucleotides. Glucose ( $C_6H_{12}O_6$ ) is the hexose mainly involved in the energetic changes of the cell. Stereochemically it can be best represented by the model proposed by Haworth (Fig 2)

The *disaccharides* are sugars formed by the condensation of two molecules of monosaccharides with the loss of one molecule of water their empirical formula therefore is  $C_{12}H_{22}O_{11}$ . The most important substances of this group are sucrose and maltose in plants and lactose in animals. All three of these sugars are derived from the condensation of hexoses. For example, maltose can be represented stereochemically by two hexopyranose rings as in Fig 2

The *trisaccharides* result from the union of three molecules of monosaccharides with the loss of two molecules of water hence their formula is  $C_{18}H_{32}O_{14}$ .

The *polysaccharides* are the result of the condensation of many molecules of monosaccharides with a corresponding loss of water molecules. Their empirical formula is  $(C_6H_{10}O_5)_n$ . By hydrolysis they yield molecules of simple sugars.

The most important of the polysaccharides are starch, glycogen and cellulose. The first two form reserve substances in cells of plants and animals, respectively. The third is the most important structural element of the plant cell.

The synthesis of starch may be brought about directly from carbon dioxide and water by means of chlorophyll (photosynthesis). Though the mechanism of this reaction is not known in all its details, recent work (Wood and Workman) showed that the first stage is the fixation of carbon dioxide by an acceptor (see Chapter V). This reaction may take place in the dark. The next stage, which requires the presence of light, is reduction to a simple sugar which, in turn, through condensation and a subsequent reduction, finally forms the polysaccharide.

In starch, two long polymer molecules are found, one, amylose, is linear while the other amylopectin, is branched. In amylose about 200 to 500 glucopyranose units are found forming a chain which can be represented stereochemically as shown in Figure 2. In the amylopectin, several glucopyranose chains are themselves joined at 1,6 linkages.

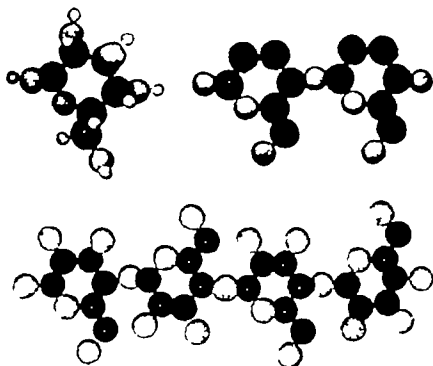


Fig. 2. Models of carbohydrates showing glucopyranose rings. *Upper left*  $\beta$ -glucose: carbon atoms in black, oxygen in gray, hydrogen in small spheres. *Upper right* two glucopyranose rings bound to form the skeleton model of maltose. *Lower*: Section of the skeleton model of a polysaccharide (amylose) (From Haworth.)

Glycogen may be considered as the starch of the animal cells. It is a polymer composed of many rings of glucose, and represents an important source of energy in the body. It is found in numerous tissues and organs, but the largest proportion is contained in the liver and in the muscle. In contrast to starch, which is found within the cells in the form of cell inclusions, glycogen, being to a certain degree soluble in water (15 to 20 per cent) may be dissolved in the protoplasm. In the living cell it is therefore invisible, but on treatment with various fixatives glycogen precipitates and can be demonstrated histochemically by means of the iodine reaction, which gives a reddish brown color with glycogen.

The content of glycogen varies according to the diet, but normally it corresponds to some 3 per cent of the total weight of

the liver. It is continually broken down and synthesized in the organism, not directly however as in the case of starch in plants, but from glucose molecules in the liver and from lactic acid in muscle (Pasteur Meyerhof Cycle). It can also be synthesized from proteins and amino acids.

*Cellulose* is another important polysaccharide in plant cells. Along with lignin, it constitutes not only the wall of the cells but also a series of other structures which form part of the supporting skeleton of plants. Cellulose is composed of units of cellobiose ( $C_{12}H_{22}O_{11}$ ) a disaccharide isomer of maltose, formed of two molecules of hexose. On hydrolysis, cellulose yields glucose.

### Mucopolysaccharides Mucoproteins and Glycoproteins (Meyer)

Under these different names there are found a vast number of compounds which are very important in the molecular organization of the cell and particularly of the intercellular substances.

*Mucopolysaccharides* are polymers of high molecular weight containing acetylated hexosamine and found free or combined with inorganic bases or with proteins. They are divided into two main groups

- 1 The *neutral mucopolysaccharides* which contain only acetyl glucosamine (i.e., chitin) or other monosaccharides like galactose, or galactose plus rhamnose.

- 2 The *acid mucopolysaccharides* which, in addition to acetyl glucosamine, contain an acid like glucuronic acid (i.e., in hyaluronic acid) sulfuric acid (i.e., in chondroitin sulfate and heparin) or phosphoric acid.

*Mucoproteins* (also called mucoids) and *glycoproteins* are complexes of acetyl glucosamine and other hydrocarbons with proteins. According to Meyer these groups differ in the amount of the amino sugar which is above 4 per cent in the mucoids and below 4 per cent in the glycoproteins. Among the first group one finds substances secreted in saliva, in the gastric mucosae, the ovomucoid and so on in the second, ovalbumin, seroalbumin, etc.

Of this heterogeneous group of substances the acid polysaccharides, and particularly hyaluronic acid, chondroitin sulfuric acid and mucoitin sulfuric acid are of importance in cytology. All three compounds are found in the ground substances of the connective tissue, where they probably act as binding and protective agents, and also in the umbilical cord.

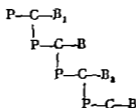
Hyaluronic acid is also found in the synovial fluid, the vitreous humor, the aqueous humor and probably in other tissues. As other high polymers, hyaluronic acid produces very viscous gels even at low concentration. This has been attributed to the presence

of long, branching particles, as evidenced by flow birefringence data and electron microscopy (Fig 126) (See Chapter XI.)

## Nucleic Acids

The carbohydrates also form compounds (symplexes) with other substances, as for instance, proteins, amino acids and so on. One group of compounds of particular interest for the cytologist is the nucleic acid group. They are fundamental substances for the cell and constitute the most important part of the chemical composition of the nuclei. In general, they form the prosthetic group of certain conjugated proteins, the nucleoproteins. The nucleic acid of nuclear chromatin may be combined with a histone or a protamine and with other proteins. Nucleic acids and their role in the cell will be treated in detail in Chapter VII and their importance in cytogenetics in Chapter VIII. However, we wish to anticipate here some basic chemical facts. The nucleic acids have a complex chemical structure; they are formed of sugars (pentoses), phosphoric acid and nitrogen bases (purines and pyrimidines). The most important are desoxyribonucleic acid, ribonucleic acid (yeast, nucleic acid) and tuberculinic acid of the tubercle bacilli.

Nucleic acids are considered to be long polynucleotides resulting from the linkage of many units called nucleotides. A nucleotide results from the combination of one molecule of a pentose with a nitrogen base (purine and pyrimidine) on one side and with a molecule of phosphoric acid on the other. Several nucleotides are bound together perhaps as represented in this diagram (see also Fig 56)



Taking a concrete example, in desoxyribonucleic acid, C is 2-desoxyribose and B<sub>1</sub> adenine (a purine) B<sub>2</sub> thymine (a pyrimidine) B<sub>3</sub> cytosine (a pyrimidine) and B<sub>4</sub> guanine (a purine). In addition to nucleic acids, several simpler nucleotides of great importance in tissue have been isolated. Among them one finds, mainly in muscle, *adenylic acid* and *inosinic acid*, both having in common one molecule of phosphoric acid, and one molecule of d ribose. They differ in the base which is adenine in the first and hypoxanthine in the second. *Guanylic acid* differs from the

former in having guanine as a base this is found in pancreas, spleen and liver. Other nucleotides have important roles in enzymatic reactions. Among them there is the *diphosphopyridine nucleotide* or coenzyme I containing adenine, nicotinamide, two molecules of d ribose and two of phosphoric acid. *Triphosphopyridine nucleotide* or coenzyme II, differs from the former in having three instead of two molecules of phosphoric acid.

## Lipids

Under this name are listed a large group of compounds which show a relative insolubility in water and a solubility in organic solvents such as benzene, petroleum ether and chloroform. This general property is due to the predominance of long aliphatic hydrocarbon chains or benzene rings. Such structures are non polar and hydrophobic. In many lipids such chains may be attached to a polar group at one end, which may then be hydrophilic, and perhaps capable of bonding to water by hydrogen bonds.

Lipids can be classified as

- 1 *Simple lipids* which are alcohol esters of fatty acids. Among these are
  - a *Glycerides* often called triglycerides, which are tri esters of fatty acids and glycerol. These are sometimes further divided into fats and oils. Fats are solid at 20°C., whereas oils are liquid at this temperature. Certain common fats are tallow, lard, human fat and cocoa butter. Among the oils are fish oils, olive oil, castor oil and the like.
  - b *Waxes* which are esters of fatty acids with alcohols other than glycerol. Beeswax is an example.
- 2 *Steroids* are lipids characterized by the cyclopenteno per hydrophenanthrene nucleus (Fig. 3). This is an aliphatic ring system, but may have one or more aliphatic unsaturated double bonds, as well as various side chains. To the steroids belong a series of highly important substances in the body such as the sex and adrenal cortical hormones, vitamin D, the bile acids and so on. Steroids possessing an —OH group are called sterols. Cholesterol is a widely distributed sterol, which is the principal constituent of lanolin or wool fat, and is found in the bile, brain, adrenal glands, and elsewhere. It often occurs in ester linkage with some fatty acid.

Stereochemically the *sterols* form complex ring systems which are rather flattened. In the case of cholesterol the molecule is

about 20 Å in length 7 to 7.5 wide and 5 Å thick. Here again is a polar —OH group at one end and a nonpolar hydrocarbon residue at the other (Fig 3)

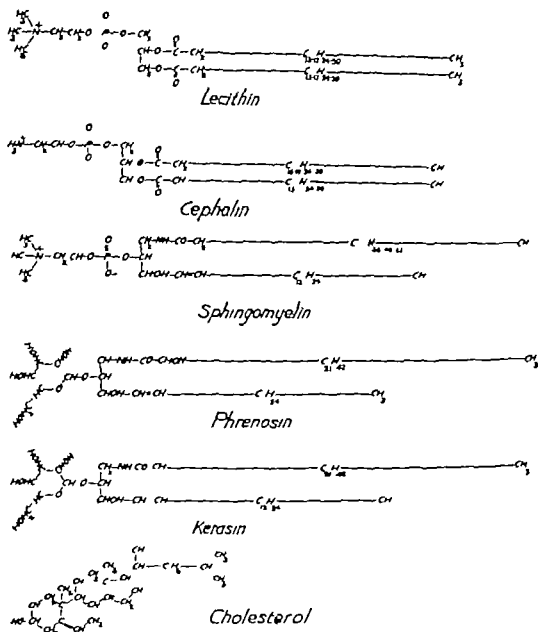


Fig 3 Stereochemical formulas of phospholipids, cerebroside and cholesterol in which the relative sizes of the molecules are maintained. (From Schmitt and Palmer)

3 **Complex lipids** are those which on hydrolysis yield other compounds in addition to the alcohol and acids. Among these are

- Phospholipids** which are fats containing phosphate and nitrogen such as lecithin, cephalin, sphingomyelin and the acetal phospholipids (Fig 3)

- b *Cerebrosides* These are fatty acids combined with nitrogen-containing carbohydrates. In this group fall kerafin, phrenosin and nervone. These are found mainly as constituents of the myelin in nerve (Fig. 3)

4 *Carotenoids* (lipochromes) are red or orange cell pigments, soluble in organic solvents, insoluble in water to which belong carotenes, in carrots and grass, xanthophyll (lutein) in leaves, vitamin A, egg yolk pigment and the like

5 *Other lipoidal substances* might be mentioned, such as the xanthocyanins, which are plant pigments and certain melanin like phenolic polymers which are soluble in organic solvents

The lipids of primary cytological interest include *triglycerides* which are composed of glycerol and fatty acids. Glycerol is a trihydric alcohol, whose formula is



It has three hydroxyl groups which can be substituted by three molecules of fatty acids so as to form a tri-ester (triglyceride). In the animal organism, the most important fatty acids found combined with glycerol are palmitic, stearic and oleic. All these acids are monovalent, three of their molecules combining with one molecule of glycerol, thus forming tripalmitin, tristearin and triolein, respectively or mixed glycerides with two or more of the types of fatty acids bound to the same glycerol residue. The fat of adipose tissue is largely a mixture of these esters in variable proportions

If a fat or oil is hydrolyzed with an alkali, the fatty acids separate from the glycerol and may form a metallic salt. This phenomenon is called saponification and the product is a soap. Stereochemically fatty acids are constituted by long hydrocarbon chains (generally containing 14 to 18 carbons) with a polar  $\text{—COOH}$  group at one end. This particular disposition of the end groups makes the fatty acids and other lipid substances highly polarized and explains the particular orientation of these substances in the presence of polar or of nonpolar solvents (See Chapter IV)

In a triglyceride three long parallel hydrocarbon chains are linked by their polar end to the glycerol (Fig. 4)

*Lecithin* can be considered as a modified fat in which one fatty acid residue is replaced by a phosphoric acid, plus a nitrog

enous base, choline. Here also are found two long parallel hydrocarbon chains of about 25 Å in length with a polar portion at one end and nonpolar groups in the other (Fig. 3) *Cephalin* is similar to lecithin but has a different base, cholamine (Fig. 3)

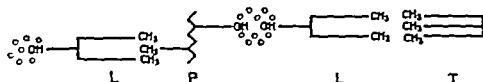


Fig. 4 Diagram showing spatial arrangement of lecithin (L) and triglyceride (T) in relation to a polypeptide chain (P) and to water molecules (O) (From Frey Wyssling.)

As in the case of the proteins, phospholipids have acidic and basic groups (*zwitterion*), in the case of lecithin the isoelectric point is about pH 6.7

The *carotenoids* are animal and plant pigments which belong chemically to the hydrocarbons and whose general formula is  $C_{40}H_{56}$ . These compounds consist of two aliphatic rings connected by a conjugated polyene chain. Carotenes can be isolated from carrots and are responsible for their orange-yellow color. They are widely distributed pigments in the plant kingdom.  $\alpha$ -,  $\beta$ - and  $\gamma$ -carotenes have been described. It is from these substances, particularly the  $\beta$ -carotene, that the animal tissues synthesize vitamin A. Another common pigment belonging to the carotenoids is lycopene, found in tomatoes and responsible for the red color of the ripe fruit.

Chemically related to the carotenoids are the *xanthophylls*, of which group lutein may be given as an example. This pigment is found in the chloroplasts of green leaves, but is overshadowed by the presence of chlorophyll. As soon as the quantity of the latter diminishes when the leaves dry out in autumn, lutein becomes manifested. Besides the carotenoids and the porphyrins (such as chlorophyll, hemoglobin and so on) the flavins have to be mentioned as biologically important pigments. In water solution they have a yellow color and a yellowish green fluorescence. To the flavins belong the lactoflavins in milk, the riboflavin (identified as vitamin B<sub>2</sub>) and so forth.

In the organism the role of the lipids varies greatly according to their location and disposition. Glycerides serve as stores of energy and in some forms (whales and walruses) may provide a protection against cold and injury. Lecithin is believed to have a role in methylation reactions in the liver (Best). Phospholipids and cerebroside are found principally in nervous tissue as con

statuents of myelin. Of the steroids, the bile acids serve as protein denaturants, and emulsifiers to aid digestion cholesterol is important in conditioning the mechanical properties of epidermis and hair and the steroid hormones regulate a number of essential metabolic and reproductive processes

From a cytological point of view it is important to differentiate the visible lipids, easily demonstrable in the cells by common methods of histochemical analysis, and the invisible or masked lipids. The former generally are visible directly in the form of refractile droplets which give readily the typical reaction for lipids blackening with osmic acid, staining with Sudan III and so on. Masked lipids, however can be demonstrated indirectly by a chemical analysis of the total tissue. For instance, in the renal cells, fat droplets are not generally observed. Nevertheless, they contain appreciable amounts of lipids. In certain pathological states, hepatic and myocardial cells show minute droplets of fat without an actual increase in the total content of lipids. This phenomenon is called lipophanerosis or fatty degeneration, a process in which part of the masked lipid becomes manifested. The invisibility of the lipids may be due to a very fine dispersion of their molecules in the cell protoplasm, or to their combination with proteins to form lipoprotein complexes (Fig 4). Typical examples of such complexes will be found in Chapters V and VI, where the structure of the membrane and of the chondriome will be discussed. There are chemical procedures which unmask some lipids and make them visible (Ciaccio). One of these consists of hydrolyzing the protein part of the protein lipid complex, and it is particularly important in the study of the Golgi apparatus (Chapter V)

## INORGANIC COMPONENTS OF THE CELL

The inorganic and mineral constituents are found in the cell in the form of salts or in combination with proteins, carbohydrates and lipids. In certain cases they may be combined with amino acids to constitute hormones (thyroxine) or with proteins to form such important compounds as hemoglobin (iron) chlorophyll (manganese) cytochromes (iron) hemocyanin (copper) and others, or with purines or pyrimidines and a pentose in nucleotides. In general, the inorganic compounds maintain the acid base equilibrium and regulate the osmotic pressure and the phosphate bond plays a very important role in glycolysis. Salts generally exist in the protoplasm dissociated into electrically charged particles, cations (with a positive charge, as for instance, Na<sup>+</sup>) and anions (with negative charge, Cl<sup>-</sup>). When considering

the distribution of substances in the body, it is important to remember that the various inorganic components are not distributed evenly throughout the tissues, but are more concentrated in some portions of the body than in others. Indeed this differential concentration or osmotic gradient may be maintained even between an aquatic animal and its environment. In Table III the concentration of certain ions in the blood of three marine animals is listed in comparison with the concentration in sea water

TABLE III  
ION CONCENTRATION IN SEA WATER AND IN THE BLOOD OF MARINE ANIMALS  
(After Macallum from Baldwin.)

	Na	K	Ca	Mg	Cl	SO
Sea Water	100	5.61	3.91	12.1	181	20.9
King Crab Limulu	100	5.62	4.06	11.2	187	13.4
Jelly fish, Aurelia	100	5.18	1.15	11.4	186	15.2
Cod C. dus	100	9.50	3.93	1.11	150	

Note that in the blood of these animals sodium is in equilibrium with that in the sea water, whereas appreciable osmotic gradients are maintained with respect to potassium and sulfate. The ionic composition of the blood of these marine animals is strikingly constant in spite of variations in the mineral content of sea water (For discussion see Barcroft, 1934 and Baldwin, 1937)

Within the organism striking osmotic gradients are also found. Thus potassium and magnesium tend to become concentrated inside cells, whereas sodium and chloride are mainly localized outside the cell in the intercellular fluid, lymph and plasma (Fig 5)

Some of the mineral substances can be demonstrated in the cells directly by using the methods of *microrincineration* (Pollicard). This consists in submitting a slice of the tissue to a temperature of about 650°C in an especially devised oven, which brings about the combustion of all the organic components of the cell. The residue which remains (ash) maintains approximately the localization which it had before incineration. The image thus obtained, compared with an adjoining slice, stained with the usual technique, may give evidence for the distribution of calcium, magnesium, iron, etc.

In addition to what was said in general about the mineral components of the cell we wish here to point out other characteristics of some of them

*Calcium* ions are found in the circulating blood, in parts of the cells and in bone and calcified cartilage, where they are engaged by phosphate and carbonate ions in a crystalline arrangement resembling that of the mineral, apatite.

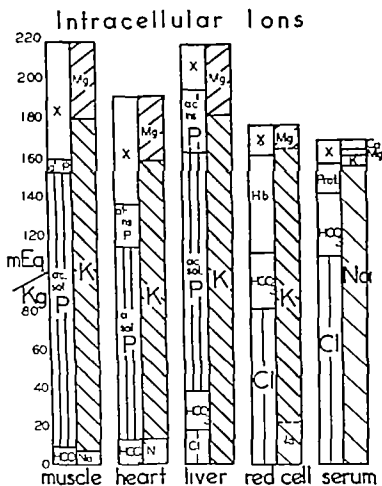


Fig. 5 Diagram showing the distribution of ions in different tissue cells in comparison to that of serum. (From Lowry)

*Phosphate* occurs in the blood and tissue fluids as the free ion, but much of the phosphate of the body is bound to organic residues in the form of phospholipids, nucleotides, phosphoproteins and the phosphorylated sugars. Phosphate tends to form anhydride linkages with other phosphates, as in adenosine triphosphate, or ester linkages with sugars or alcohols, as in nucleotide and lecithin. It is important in anaerobic glycolysis, where the phosphate anhydride bond is an important source of energy. In plant cells it has a role in the formation of the cell wall. As primary phosphate ( $\text{H}_2\text{PO}_4^-$ ) and secondary phosphate ( $\text{HPO}_4^{2-}$ ) it contributes to the buffer mechanism stabilizing the pH of the blood and tissue fluids.

*Chloride* circulates in the blood and intercellular fluid as the ion, and is found in much smaller quantities in cells. It is a component of gastric juice, where it occurs as hydrochloric acid. Together with sodium, it is important in osmotic regulation of cells and in maintaining globulins in solution.

*Potassium* is present in high concentration within the cell cytoplasm. Muscle contains about 300 mg. of K per 100 gm. of wet tissue, whereas the erythrocyte contains as much as 400 mg. per 100 gm. It is believed to have an important physiological part in nerve conduction and muscle contraction.

Other ions found in tissues are sulfate, carbonate, bicarbonate, magnesium and amino acids.

Certain mineral components are likewise found in un-ionized form. Thus iron, bound by metal-carbon linkages, is found in hemoglobin, ferritin and in the cytochromes.

*Sulfur* is found in the amino acids cysteine, cystine, and methionine, where it is bound by covalent linkage to carbon. The tripeptide glutathione which contains a cysteine residue, is a widespread metabolite in active cells. Sulfur in the sulfhydryl group is found in the myosin of skeletal muscle and certain essential enzymes and is important in contractility. Sulfur is also found in the sulfate ion in the plasma, and in intercellular fluids.

In order for the cell to maintain its activity, it is necessary that there exist in the medium a well balanced equilibrium of different ions. If for instance, the heart of a frog is perfused with a saline solution containing only one salt (say NaCl) it beats for a time, but stops after a little while. If, however, other salts are present in proper amount the beating persists for a much longer time. For these reasons, the artificial physiological solutions (such as Ringer's or Tyrode's solutions) are not only isotonic (have an osmotic pressure identical with that of the blood and tissues), but must embody a proper ionic balance.

## PHYSICOCHEMICAL ORGANIZATION OF THE PROTOPLASM

### Colloids

From the physicochemical point of view protoplasm can be regarded as a complex colloidal system as such it possesses some of the characteristics and properties of the colloids.

In 1861 Thomas Graham divided chemical substances into two classes. In one he included substances like salts, sugars and so on which dissolve rapidly in water and can easily pass through semipermeable membranes (see Chapter VI) while in the other group he placed such substances as the proteins, gums and so on, which form suspensions and do not cross the membranes.

The substances in the first group he classified as *crystalloids* (since the majority of them are crystalline) whereas those of the second group he called *colloids* (Gr *Colla*, gum) hoping that this division could be applied to all existing chemical compounds.

This concept was based on the erroneous assumption that colloids are amorphous. Modern investigations have revealed that many colloidal particles like those of certain proteins, enzymes and even viruses (Fig 1) may have a regular structure and may aggregate in crystals.

In modern usage one may apply the term "colloid" to a system which involves particles or aggregates ranging in size from about 1  $\mu$  to 100  $\mu$ . These limits are arbitrary and have no special significance in themselves. Substances are spoken of as being in the colloid state when aggregated in particles of a size falling within the colloid range. Such aggregates may be dispersed in a fluid, gas, or solid. In some cases the dispersed particles may show some of the properties of solutions in other cases they behave like suspensions. In neither case can the aggregate pass through an ordinary dialyzing membrane permeable to ions and small crystalloids. Particles of colloidal size may bear an electric charge and migrate in an electric field, and hence behave like ions and carry an ionic current.

In Table IV are given the dimensions of some aggregates of colloidal size and their position with respect to the size of other particles.

TABLE IV

0.1 millimicron (0.1 $\mu$ )	0.0000001 mm (1 $\text{\AA}$ )
1 millimicron (1 $\mu$ )	0.000001 mm (10 $\text{\AA}$ )
1 micron (1 $\mu$ )	0.001 mm (10,000 $\text{\AA}$ )
Cells and bacteria	10 $\mu$ –0.1 $\mu$ (100,000–1,000 $\text{\AA}$ )
Colloidal particles	1 $\mu$ –100 $\mu$ (10–1,000 $\text{\AA}$ )
Particles of colloidal gold	1.7 $\mu$ (17 $\text{\AA}$ )
Starch molecule	8 $\mu$ (80 $\text{\AA}$ )
Hydrogen molecule	0.1 $\mu$ (1 $\text{\AA}$ )

In a colloidal system, particles or aggregates constitute the so-called *dispersed phase* which is suspended in the *dispersion medium*. For example, in the case of a colloidal gold, the gold particles form the dispersed phase whereas the water is the dispersion medium. Some of the properties of colloidal systems have been known since 1838 but their significance was understood only later. In that year Robert Brown, the botanist who three years earlier discovered the nucleus, observed under the microscope that grains of pollen undergoing germination were in constant movement. This motion is now called 'brownian move-

ment, and is due to the collisions of molecules of the dispersion medium against the colloidal particles

The brownian movement depends on the size of the particles and the viscosity of the medium and is proportional to the temperature. The higher the temperature of the liquid, the more rapid is the thermal agitation of the molecules and therefore the more frequent the bombardment of the particles

When a beam of light passes through a colloidal solution, the path of the light becomes visible. This is due to the so-called Tyndall effect which is produced by the scattering of light from the surface of the colloidal particles. This phenomenon is similar to that frequently observed in a dark room when a beam of sunlight enters through a tiny hole and particles of dust which can hardly be seen in the diffuse light become visible through the scattering of light on their surfaces. Similarly, the blue of the sky is a result of the scattering of light in the atmosphere, and is an example of the Tyndall effect. Also the blue of the eyes is due, not to a pigment, but to the scattering of light in the sense of a Tyndall phenomenon

The phenomenon of light scattering is the basis of the *ultramicroscope* or *darkfield microscope* of Siedentopf and Zsigmondy which permits one to observe the location of colloidal particles by noting the position of the points of light which they scatter. In the ordinary microscope, the light traverses the condenser and the object vertically. In the ultramicroscope the beam of light enters from the side and is reflected by the particles. The observer sees the reflected point of light, which is usually in noticeable brownian movement (Fig. 6)

Light scattering by colloidal particles depends on the intensity and the wavelengths of the incident light, the number and size of the particles, and the difference between the indices of refraction of the medium and the particles. When the indices become equal, as in the case of some hydrophilic colloids, the Tyndall phenomenon disappears. Protein colloids generally show no marked Tyndall effect in visible light, although they may scatter ultraviolet light.

The fact that the colloidal particles can be maintained in suspension without being precipitated suggests the existence of a mechanism which interferes with the influence of gravity. This can occur if the suspended particles are electrically charged. If one passes an electric current through the colloidal solution the particles may be displaced either towards the anode or towards the cathode. By means of this phenomenon of *electrophoresis* it is possible to demonstrate that particles of hemoglobin, copper

and so forth are positively charged, whereas colloidal gold is charged negatively

Often colloidal particles may be considered as surrounded by two electrical layers of opposite charge (Helmholtz double layer) an internal one, which adheres very strongly to the particle, and an external one, labile and composed of hydrogen ions ( $H^+$ ) if the internal layer is of negative charge, or of hydroxyl ions ( $OH^-$ ) if the internal layer is of positive charge. The particles as such

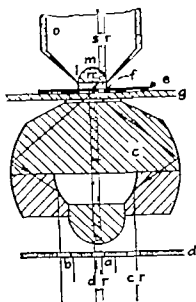


Fig 6 Spenser's objective and cardioid condenser for darkfield work o, object e f frontal lens of the objective m, silver mirror (Spencer) rr rays reflected from the mirror e, coverglass f material to be analysed, g slide c, cardioid condenser d, fixed diaphragm, a, aperture of 1.5 mm. for light directed to the Spenser's lens (d) opening for the rays which go to the cardioid condenser (c) (After Seuffert)

may therefore be neutral. If, however an electric current passes through the colloidal solution, the particles with their strongly adhering layer slip out from the external layer and wander toward the anode or the cathode, according to the internal charge. In the case of gold suspensions, the internal negative layer is surrounded by a positive shell of hydrogen ions. If the particles of colloidal gold are separated by a sufficient distance (i.e. greater than the diameter of the particle) there is no strong electrical influence exerted between them but if by action of brownian movement they approach each other closely the outer layers of the same sign cause the particles to be repelled. In this way an agglomeration of the particles can be prevented. If the size of the particles is sufficiently small, they may be sufficiently moved by the bombardment of the molecules of the solvent to cause the suspension to remain stable for an extremely long

time. This labile electrical charge, represented by the zeta potential, therefore plays an important role in the stability of colloids, particularly the hydrophobic colloids. In many colloidal systems there are likewise attractive forces between particles which may tend to cause aggregation and to complicate the picture.

Although the size of the colloidal particles is of great importance, this does not signify that they are necessarily composed of aggregates of atoms, as in the case of colloidal gold or of aggregates of molecules. There are, for instance, protein molecules of such large size that the dimensions of a single one fall within the range of colloidal particles and, when dispersed in water, the solutions show the characteristics of colloidal systems.

One can list eight kinds of colloidal systems. They are shown in Table V along with typical examples of each.

TABLE V  
TYPES OF COLLOIDAL SYSTEMS

Type of system	Example	Dispersed Phase	Dispersing Phase
1 Liquids in liquids (emulsions)	Milk	Fat	Water
2 Solid in liquid	Gold sol	Gold	Water
a Hydrophobic (suspensoids)			
b Hydrophilic (emulsoids)		Protein	Water
3 Solid in solid	Ruby glass	Gold	Glass
4 Liquid in solid	Pearls	Water	Calcium carbonate
5 Liquid in gas	Fog	Water	Air
6 Solid in gas	Smoke	Carbon	Air
7 Gas in solid	Charcoal	Air	Carbon
8 Gas in liquid	Foam	Air	Water

Of these eight types of colloidal systems, the ones of principal biological importance are the emulsions (Table V 1) and emulsoids (Table V 2b). The suspensoids have been described as examples in the general consideration of the colloids.

The most important characteristic of the emulsions is that, although both phases are liquids, the dispersed phase does not mix with the dispersion medium. For this reason they are called lyophobic colloids. To this group belong the mixtures of oils and fats in water as well as water in oils, and so forth. They are much more complicated than they appear to be, because in order to maintain the hydrophobic particles in suspension, the presence of a stabilizing agent is usually necessary. These stabilizers are principally hydrophilic particles which form a film around the hydrophobic particles, thus constituting a layer with affinity for the solvent (for example, gelatin solution added to an oil water

system) Jacques Loeb who studied the mechanism of the phenomenon, called such substances protective colloids. Such emulsions are found in protoplasm when small droplets of oil or fat become suspended in an aqueous phase, stabilized by a protective colloid. They can sometimes be seen with the ultramicroscope. For example, fine particles called *chylomicrons* are seen in blood plasma after meals (Ludlum, Taft and Nugent). If, for any cause, the protective mechanism of the colloid system does not work properly, the dispersed phase may aggregate. The formation of gallstones is supposed to be due to such a phenomenon.

*Emulsoids* are important colloids from a biological point of view. In the emulsoids, unlike the suspensoids there is a mutual attraction between the two phases. To this group belong, for instance, all the protein suspensions in water as well as those of some carbohydrates. A typical example of such a colloid is a solution of agar-agar in water. The molecules of water penetrate between the molecules of agar-agar causing a swelling of the latter. By adding successively more and more water a colloidal solution is finally obtained in which the dispersed phase is composed of hydrated particles of agar-agar. Since in this case the dispersing liquid is water and there is an affinity to take it up, such colloids are called hydrophilic, in contrast to the hydrophobic. Hydrophilic colloids are much more stable than the hydrophobic ones. This stability is attributable in part to the electric charges on the particles and in part to the attractive action of the dispersing medium, which forms a kind of a halo of water (called the layer of solvation or hydration) around the particles.

In this layer the water molecules are oriented and polarized by the electrical charge of the particle. At the isoelectric point, the charge of the particle may be at a minimum, whereupon the layer of solvation will also be reduced to a minimum and precipitation may occur. On the other hand, at a pH far from the isoelectric point the charge is usually greater and the shell of oriented water molecules is larger (Fig. 7). Between the oriented bound water molecules and the free ones there is a continuous gradation as the forces binding them to the particle decrease exponentially with the distance. Because of this gradation in many hydrophilic colloids, no real interface between the particles and the dispersing medium exists, and the stability of the system is great. In protoplasm many substances are hydrophilic and are

In water molecules electrical charges are arranged asymmetrically in space, forming a dipole, since the two positive hydrogens are separated from the double negative oxygen (Fig. 7).

found in this type of colloidal state. As mentioned already, the most common substances in this category are certain carbohydrates and proteins

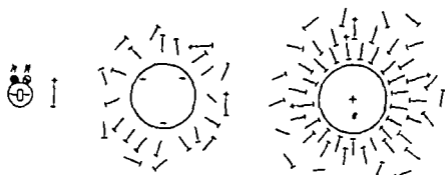


Fig. 7 *Left*, model and dipole diagram of a water molecule. *Middle* diagram of the hydration of a colloidal particle at the isoelectric point. *Right* hydration of a positively charged colloidal particle. (From Frey-Wyssling. *Right* after Pollmann)

### Coacervates

We have seen that there are two factors—electrical charge of the particle and solvation layer—which tend to stabilize emulsoids. When one or both of these factors are changed, particles tend to coalesce and to form what is called a coacervate.\*

Coacervates are classified as simple or complex (Bungenberg de Jong). *Simple coacervates* result from the removal of water from the layer of solvation by a substance in molecular dispersion or by another hydrophilic colloid of the same charge. Among the water removing agents, aliphatic alcohols or acetone is generally used. As the solvation layer diminishes it becomes less diffuse and the boundary between bound and free water becomes more marked. The surface energy which was before at a minimum now increases, and the water shells coalesce bringing the particles close together in a condensed system (Fig. 8). In the coacervate the solvation layers are fused but the particles are still separate

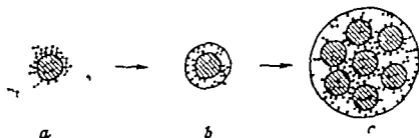


Fig. 8. Diagram of the process of coacervation. *a*, colloidal particle with diffuse hydration layer *b* hydration layer reduced and delimited *c*, beginning of the coacervation phenomenon (see description in the text) (From Bungenberg de Jong)

Coacervation. Latin, *Acervus*, heap or swarm; with the prefix "co" (together). Between coacervation and flocculation there is only a difference of degree

from one another. The dispersion medium separating from the coacervate is called equilibrium medium.

*Complex coacervates* result from the action of polyvalent ions (i.e.,  $\text{CaCl}_2$ ) or from the interaction of two hydrophilic colloids of opposite charge (i.e., gelatin and gum arabic). This last type of coacervate seems of great importance in biological systems since colloidal systems of opposite sign are frequently found in protoplasm. So for example, basic proteins (with an isoelectric point above pH 7) like histones and protamines, may form complex coacervates with acid proteins, nucleic acid, phosphatids, chondroitin-sulfuric acid and so forth.

Coacervates may show morphological appearances resembling certain cellular inclusions or organoids like vacuoles, lipid droplets, chondriosomes, or Golgi apparatus and the theory of coacervation can be used to some extent to explain the formation of such intercellular entities (see Chapter V).

### Tactoids

Tactoids are another type of colloidal structure resulting from the interaction between particles (Zocher). Under the polarizing microscope (see Chapter IV) tactoids appear as spindle shaped bodies which show intense birefringence. This phenomenon indicates that tactoids are built of elongated particles oriented along their main axis. One of the best examples of tactoids in biological material is represented by the tobacco mosaic virus (TMV). The elongated virus particles (Fig. 1) may pack together and orient themselves to form tactoids (Bernal and Frankuchen). The distance between the particles is very constant and in TMV may range between 150 Å and 300 Å, according to the salt concentration. However in other kinds of tactoids it may reach up to 5000 Å. This large interparticle distance may indicate the action of long range forces (see Chapter IV).

Tactoids can be considered as a special kind of coacervate with anisodiametric particles. As in the case of coacervates, tactoids have also been compared with some cellular structures like the spindle, and a role in cellular processes has been claimed (Bernal).

### Gels

Hydrophilic colloids in concentrated solutions may show mechanical properties (viscosity, elasticity, tensile strength, and the like) which differ from those found at lower concentrations. In the first case, the colloid forms a gel while in the second it is called a *sol*. A typical example is that of gelatin which forms a

liquid solution (sol) by dissolving in hot water, but when allowed to cool off may form a highly viscous and elastic gel. Many other proteins, certain polysaccharides and other polymers may likewise form gels when dissolved in the proper concentration.

Gels are frequently liquefied (transformed into sols) by changes in temperature (such as in the case of gelatin) pH, salt concentration, pressure and so forth. This change, called *solation*, may sometimes be reversed again by removing the factor involved. In this case a *gelation* takes place.

To explain the mechanical properties of gels one has to suppose the presence of a marked interaction between the dispersed colloidal particles. Such an interaction can take place in the case of spheroidal particles, but in that case a high concentration is necessary. In contrast, elongated particles of molecular chains can interact even at low concentrations (Fig 9 a)

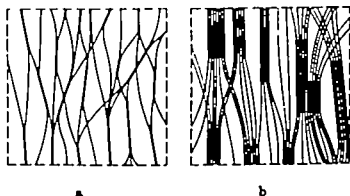


Fig. 9 Diagram of gels of long polymeric particles *a*, with some parallel orientation of the particles, and *b* with sites of crystalline structure. (From Frey Wyssling.)

It is now known that most of the gels are formed by long polymer chains which form a kind of three dimensional network or spongework or 'brushheap' by means of attractive forces at the points of intersection of the chains. The shapes of polymeric particles can often be ascertained indirectly by means of viscosity studies, determination of birefringence of flow diffusion, sedimentation, or directly by electron microscopy. Figures 126 and 138 show the shape and size of the particles in the case of sodium hyaluronate and actin gels. The first shows branched filaments, while the second shows very long straight particles. This last figure shows a typical solation of a gel by changing the pH, filaments are broken down into short rod shaped particles.

The bonds involved in cross linkages may vary from covalent or salt linkages to hydrogen bonds and van der Waal forces. The varying strength of these bonds, together with characteristics

of the particles, may explain the stability of certain gels and the easily reversible character of others. For example, gels formed by three dimensional polymers with branched chains, may be very stable and irreversible. On the other hand, gels depending on hydrogen bonds or van der Waal forces, or made up of particles which may change in length, are less stable and are easily reverted to sols. Because chainlike particles are generally of molecular dimensions, gels really form a monophasic system in which water is contained in the spaces of the framework. For this reason gels are generally optically homogeneous and show no Tyndall effect. This particular disposition of the water in relation to the framework of particles explains why colloids containing 80 to 90 per cent or even more of water may form gels.

In a gel, particles are generally oriented at random so that the gel is isotropic. However in some cases, particularly under the influence of stress, the particles may tend to become preferentially oriented parallel to each other and to render the medium anisotropic. When this process is more advanced, the formation of crystalline regions inside the gel may take place. (Fig 9 b) This process of crystal formation is particularly important in the morphogenesis of some cellular and intercellular structures (See Chapter IV)

Some reversible gels may show a phenomenon of reversible solation-gelation with striking changes in viscosity under the influence of mechanical forces. This attribute is called *thixotropy*\* and is seen, for example, in certain paints which become less viscous when agitated with a brush but again more viscous when left standing. The forces acting between the particles and causing the phenomenon of thixotropy are not well known. Apparently thixotropic gels are closely related to coacervates and tactoids and may depend on long range forces (Freundlich). The asymmetry of the particles, the electrolyte content of the dispersion phase, pH and temperature are also important in this process.

*Protoplasm* can be considered to be a heterogeneous colloidal system made of a framework of filaments, membranes, microsomes, and so forth, which are dispersed in a watery medium. The components of this framework are long molecular chains or molecular aggregates called *micelles* † and the cross linkages between the micelles are supposed to change constantly according to local metabolic changes (Seifriz, Frey Wyssling). In several

Greek: *Thixis* a touch and *Tropes*, to change.

† This term was used for the first time by the Swiss botanist Nägeli who, in the second half of the nineteenth century proposed a similar structure of the protoplasm.

chapters of this book examples of the behavior of protoplasm as a thixotropic gel will be described. Here we will mention only the action of hydrostatic pressures on the protoplasmic gel of the cell. When moderate hydrostatic pressures (5000 pounds per square inch) are applied to cells, a group of physiological activities which are related to solation-gelation changes of the plasma gel, like cyclosis, amoeboid movement, cell division and migration of pigment in chromatophores, are completely inhibited. This inhibition is due to the degree of solation which the pressure induces in the plasmagel system (Marsland).

Another property of gels is their *contractility*. On contracting, gels expel a part of the liquid phase along with the dissolved substances in the latter. This rather common process is called *syneresis*. Typical examples of syneresis are the expulsion of water produced by the retraction of the coagulum of milk, or the extrusion of serum from clotted blood. Some authors suppose that the mechanism of cellular secretion is due to the contraction of the protoplasmic gel.

Protoplasm has the property of absorbing and eliminating water. This might be, along with other factors, a reason for its continuous changes of viscosity. If for instance, an amoeba is observed with the ultramicroscope, the brownian movement of the colloidal particles in its body is easily distinguishable. This movement is sometimes rapid, sometimes slow or it may stop. Such behavior indicates changes in the viscosity of the protoplasm. According to W. H. Lewis (1942) amoeboid locomotion depends, among other things, upon the local changes of viscosity: gel layer into fluid or semifluid endoplasm, and endoplasm into gel layer. (See Chapter XI.) It has also been stated that, in cell division and cleavage, there are continuous changes in viscosity.

The absorption and loss of water from the protoplasm has a great significance in vital processes. Thus in muscular contraction, there is probably a change in water distribution inside the fiber. The motion of many plant cells is, in all probability, due to similar causes. For these reasons a deeper knowledge of the mechanism of solation-gelation and other phenomena related to it will have great importance for a better understanding of the processes of life.

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### Chapter III

## MORPHOLOGICAL ORGANIZATION OF THE CELL

In Chapter I we described the cell or *protoplast* as a small mass of protoplasm with a nucleus, surrounded by the plasma membrane. The protoplasm which envelops the nucleus is called, in general, *cytoplasm* and in the case of each particular cell, *cytosome* (Fig 10)



Fig 10 Photomicrograph of an ovocyte of a mammal. Example of a very voluminous cell (compare it with the size of the surrounding follicular cells) with a clear vesicular nucleus provided with a nucleolus, chromatin filaments and a karyotheca or nuclear membrane. The cytoplasm is granular and is surrounded by a thick membrane (pellucid) which is not the true plasma membrane. Hematoxylin and eosin.

In a multicellular organism the cells have a form and structure which is extremely varied, conditioned principally by the adaptation to the specific function which they carry out in the different tissues and organs. The functional specialization, which results from the division of labor causes the cells to acquire special characteristics in each case but always some attributes persist which are common to all of them. These general characteristics, which are those treated preferentially in this book, can be found in cells but little differentiated, such as the blastomeres, or germinative cells, and also in cells with a relatively simple organization, such as those of the epithelium or of the connective tissue.

The study of cellular morphology can be made in direct form in the living organism or immediately after isolation of the cells. This is the *immediate examination*, which may be *vital* or *supra vital*. The tissues may be also submitted to special procedures which kill the cell and preserve its morphology and composition. This is the *mediate examination* or *postfixation examination*.

The vital or supravital examination is applied to free cells in a liquid medium, to cells isolated from fragments of tissue, to transparent cellular membranes, to transparent animals or parts of transparent animals (larvae of urodeles) and even to opaque organs. In the last cases, there have been perfected a series of biomicroscopic procedures, such as grafts in the anterior chamber of the eye, transparent chambers which are installed in the ear of the rabbit (Sandison-Clark) the epicondenser and illumination with quartz rods (Knisely) which have extended considerably the field of vital observation of cells and tissues.

The main difficulty in biomicroscopy arises from the fact that the different parts of the living cell generally do not absorb visible light (see Chapter VII). Although the differences in the index of refraction of various structures may be sufficient to produce phasic changes these cannot be appreciated by an ordinary microscope. The result is that living structures show very little contrast and hence are difficult to see under the microscope. In recent years this problem has been partially overcome by the use of methods in which contrast is increased by special optical means. In one of these methods, the *phase contrast microscopy* (Zernike) phase differences of the various structures are converted into differences in amplitude appreciable to the eye. For this reason, phase contrast microscopy is a valuable tool in the observation of living cells. With this method the nucleus, the nucleolus, the chromosomes and, in certain cells, even the Golgi apparatus can be clearly seen and photographed because of the increased contrast obtained.

One of the methods which permits us to observe the cells, not only in a state of mere survival but under more favorable conditions, and also to follow their development is *tissue culture*, originated by Harrison and developed by Carrel. This technique consists in explanting small portions of different tissues, preferably embryonic, in a suitable medium, where the cells can adapt themselves and grow in an autonomous form. One generally takes small pieces or parts of the embryo chick (or other tissue) and places them in a medium consisting of one drop of plasma and another of embryonic juice, which is deposited on the surface of a coverglass. Then this is inverted on a special slide, provided

with an excavation, and is bordered with paraffin. In this closed chamber, which should be perfectly aseptic and maintained in an incubator at the temperature of the animal body, the cells have the nutritive elements and the oxygen necessary for their development.

The cells grow and spread over the coagulum of plasma and pass out from the explant to form the *zone of growth* which, by its thinness lends itself admirably to the vital observation of the cells (Fig 11)



Fig. 11 Tissue culture of muscle cells. The dark zone below is the explant, out side of which springs the zone of growth and migration where the cells can easily be observed *in vivo*. (From a preparation of Dr Sacerdote de Lustig)

To maintain the cells in optimum vital conditions during an indefinite time, it is necessary to replenish the nutritive elements, eliminate the cellular wastes and, when the growth reaches a certain limit, to transfer it. This consists in taking out a small piece of the culture and explanting it anew in the same form as at the beginning

### GENERAL MORPHOLOGY OF THE LIVING CELL

Whether we observe them isolated in isotonic liquids (blood serum, aqueous humor Ringer's fluid, and so forth) or in tissue culture, the cells appear as irregular masses, translucent and with a paler sphere within their interior the nucleus. Between the two parts of this heterogeneous system, the cytosome and the

nucleus, there is found the *karyotheca* or *nuclear membrane* which is generally visible with a darkfield microscope as a fine limiting line (Fig 12)

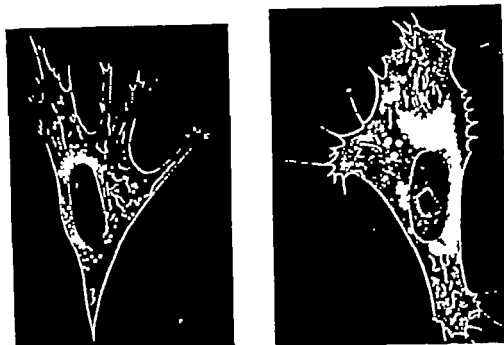


Fig 12 Connective tissue cells cultivated in vitro. Vital observation in darkfield. The nuclear membrane, nucleoli, chondriosomes and droplets of fat are seen. (From Strangeways and Cantu)

### Plasma Membrane

The surface of the cytosome is considered to be surrounded by a plasma membrane which is involved in the regulation of the permeability of the cell. This membrane is an essential attribute of every protoplast and is found even in protoplasmic masses such as bacteria and Cyanophyceae, the cellular nature of which is open to question. Although it has been demonstrated with special apparatus (such as the leptoscope, Chapter VI) in the case of the erythrocyte, it is in general invisible with the microscope. Its thickness was found to be about  $0.010 \mu$ , and thus is below the limit of microscopic resolution. The plasma membrane of the erythrocyte has also been studied by means of the electron microscope (Wolper).

In Chapter VI we shall study in extenso the constitution and properties of this specialized part of the cytosome. Here we shall only say that it appears to be composed of bimolecular layers of lipids, oriented perpendicularly to the surface, between which are found flat layers of protein molecules of an extended shape.

This membrane, of such delicate structure, lacks a strong

mechanical resistance, for which reason it is reinforced externally by others more coarse and resistant. These are the membranes which are in general visible to the microscope and which should not be confused with the plasma membrane, which is invisible.

In these external layers the carbohydrates appear to be one of the essential components. Indeed, about the eggs of marine animals, such as the sea urchin, gastropods, worms, and those of amphibia, there is a gelatinous substance, *mucin*, which is a glycoprotein. A similar protein conjugated with an amino sugar polymer is found capping and protecting the cells of the gastro-intestinal tract. Other modified carbohydrates are *pectin* and *cellulose* of the membranes of plant cells and *chitin* of the Crustacea.

In the space situated between the membranes of different cells of the higher vertebrates, there appear to exist glycoproteins in addition to simple proteins. The adhesions between the cells of kidney epithelium, of the endothelium of the capillary and other cells, appear to depend on a cementing substance produced by the cells which requires the presence of calcium ions (Chambers-Zweifach). Recent studies demonstrate that simply by the addition of small quantities of *histone*, the red cells adhere in masses which resemble epithelia, morulas, and so forth. This demonstrates the mechanical and morphogenetic importance of these protein films situated outside of the plasma membrane (Schmitt).

## Cytoplasm

Cytoplasm appears as a translucent, structureless, homogeneous substance, optically empty to the ultramicroscope, in which are found refractile bodies of varying size (Fig. 12). The homogeneous mass itself is called the *fundamental*, or *basic*, or *ground* cytoplasm, and also *cytoplasmic matrix*, or *hyaloplasm*. Frequently the more peripheral layer of the cytoplasm, the *ectoplasm* is relatively more rigid and is lacking in granules. This zone often behaves as a thixotropic gel, that is, a colloid containing large molecules which has the property of undergoing reversible changes of gelation and solation. This thixotropic transformation, which is very evident in amoebae during the extension of the pseudopods, is in reality a general mechanism which is found in every cell (Fauré-Fremiet). The internal cytoplasm or *endoplasm* has a lesser viscosity but always higher than that of water (two to ten times).

If the eggs of the sea urchin are submitted to an intense and prolonged centrifugation, the different components of the endoplasm become stratified in accordance with their densities and the ground cytoplasm is separated from the other components (Fig. 13). The egg is first elongated and next becomes constricted in the central part. In the centripetal pole there are accumulated the drops of fat; there follow a clear and wide zone, the cytoplasmic matrix, which contains the nucleus; a layer occupied by the mitochondria; another with yolk bodies; and finally in the centrifugal pole, granules of pigment. It is interesting that the

most peripheral layer of the cytoplasm, or the *cortex*, is not displaced by the centrifugation, a fact which is due to its greater viscosity and rigidity. This last property appears to depend on the presence of the calcium ions, since the cortex liquefies when eggs are treated with an oxalate (Heilbrunn)

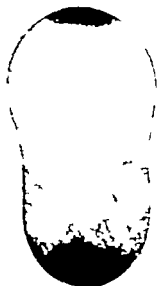


Fig. 13 Sea urchin egg (*Arbacia*) submitted to the action of centrifugal force. The egg has elongated and is in the process of being divided into two halves. The cellular materials become stratified (see the description in the text) (Courtesy of Costello.)

All the modifications produced in the eggs of the sea urchin and in other cells by the influence of centrifugal force which proceed up to the complete stratification of their components can be followed under the microscope by making use of the apparatus originated by E. B. Harvey in which centrifugation is effected exactly in the focal plane of a compound microscope.

We have seen that in the midst of the protoplasmic matrix there is a series of bodies or particles of varying size which are distinguished by their greater degree of refractivity. Among these are found the chondriosomes, which appear as spherical bodies (*mitochondria*) or elongated in the form of a rod or filament (*chondrioconts*) which, because they are constant and essential elements of the cells, are included in the category of the organoids (Chapter V Fig 12). There are also smaller bodies (*microsomes*) which have been isolated very recently by ultra centrifugation and which, although they are at times visible, in general are of submicroscopic dimensions (see Chapter IV).

Other bodies which can be observed with the immediate examination are different inclusions, such as drops of fat, which are recognized by their refractile properties, volk bodies, pigment,

secretion granules, and so forth (Fig 12) The majority of these particles are material elaborated by the protoplasm, constituting in their entirety the *deutoplasm*, or *paraplastm*.

In plant cells *plastids* of various types are found. Among these are the *chloroplasts* which contain a green pigment, the chlorophyll, the function of which is *photosynthesis*: a process of immense importance in the biological world In addition there are the *leucoplasts* or colorless plastids, which under certain conditions can be converted into chloroplasts or into plastids of other colors (chromoplasts) or store up starch (amyloplasts) or oils, or perform other functions

Both in animal and plant cells, but more commonly in the latter there may be found vacuoles of fluid content, surrounded by a membrane When vacuoles, plastids, or mitochondria are isolated from the cell, they expand or shrink in accordance with changes of osmotic pressure, obeying the law of Boyle-Marriott. These phenomena might depend on the existence of interface membranes which regulate the osmotic interchanges

These bodies will be described in detail in Chapter V Here we shall occupy ourselves with further study of the characteristics of the basic cytoplasm or cytoplasmic matrix which is, in reality the most important part of cytosome A great part of the knowledge of the properties of the matrix of the living cell is due to the employment of the *technique of micromanipulation* or *microsurgery* This method had its origin in the field of bacteriology (Schouten, Barber) where fine micropipettes were employed to isolate microbes and to transport them to culture media Hite applied this method for the first time in the domain of cytology and in 1911 interposed a microneedle between the two pronuclei of a recently fertilized egg and observed that they acted as if attempting to overcome the interposed resistance and complete their conjugation

*Microsurgery* consists in the introduction into the cells and tissues of micropipettes, microneedles, microelectrodes, microthermocouples, and so forth, with the aid of special apparatus which permits the controlled movement of these instruments in various directions under the field of a compound microscope. With this instrument one can carry out (1) the dissection of parts of cells (2) extraction of parts of cells or tissues (3) the injection of substances and (4) the measurement of electrical variables (Fig 14)

On penetrating with a microneedle into the cytoplasm of a cell, one can recognize that the peripheral part has a denser consistency and, further that the different visible structures have

a differing stability. Thus the mitochondria or the fibrillae can be displaced without being altered, but the aster disappears if displaced. The fundamental cytoplasm behaves like a reversible sol-gel colloidal system. By the mechanical action of the needle one can produce a rapid gelation on the part of the cytoplasm or of the nucleus which generally is reversible (thixotropic reaction) or indeed, gelified zones can be transformed into sols of liquid consistency.

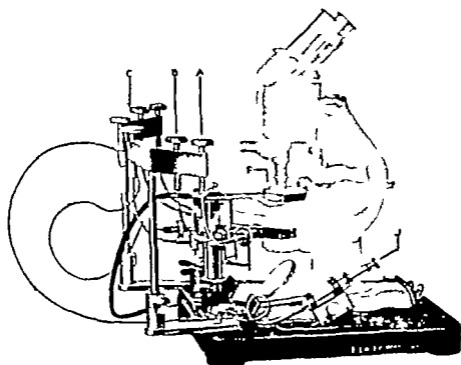


Fig. 14 Micromanipulation apparatus of Chambers. *A* and *B* screws which move the microneedles in the horizontal plane; *C* screw for vertical movement, *F* moist chamber where the cell or the tissue is located and where the microdissection is carried out, and *J* syringe for microinjection.

This method is one of those which permits us better to demonstrate the existence of a plasma membrane that regulates the interchanges of the protoplasts with the medium, although it is not visible to the microscope. Thus, if a coloring material, which because of its molecular size cannot cross the membrane, is injected, it diffuses through all the cytoplasm, but remains contained in its interior by the barrier formed by the plasma membrane (Chambers). If, with a microneedle, one breaks the superficial layer of protoplasm (plasma membrane) the behavior of this varies according to the extent and rapidity with which the lesion is produced. If the destruction is of small extent, it is repaired with facility and the plasma membrane is reconstructed. In the case of more drastic injury the endoplasm may flow to the outside, whereupon the cell dies.

An ingenious method to study this superficial cortex of cytoplasm consists in bringing close to a cell a micropipette loaded with oil and then expelling a small drop until this is placed in contact with the surface of the cell. The cell and the oil behave like two drops of liquid in contact and there is produced a phenomenon of coalescence by which the drop of oil is rapidly incorporated in the cellular cytoplasm. This method permits one to demonstrate the existence of tangential

rigidity on the surface of the cytoplasm, which varies in different functional states or through the action of different factors. Indeed, the greater or lesser facility with which the coalescence is produced depends on the surface tension in the water-oil interface and on the diameter of the drop, and the potential energy of the oil must be greater than that of the superficial cytoplasm in order that coalescence may be produced (Kopac)

The injection of pH indicators, the color of which changes with the concentration of hydrogen ions in the medium, permits us to determine the pH of the cytoplasm and of the other parts of the cell. It is not known with certainty what is the phase which is vitally colored with the indicators, but probably it is the aqueous medium of dispersion. The cytoplasmic matrix has a weakly acid reaction (pH 6.7 to 6.9) which can be better determined after centrifugation of the cell but certain vacuoles of the cytoplasm can have as low a pH as 5.0

Characteristic of the protoplasm is its buffering power. The pH of the cell can be altered by the addition to the medium of acids or alkalis or by the injection of the same, but it rapidly returns to its former value, as long as the vitality of the cell is not altered (Ries)

The oxidation-reduction potential (or reducing ability of the cytoplasm) can be determined by introducing into the cell certain dyes which have the property of changing their color or of being decolorized when reduced by the cell. This property is of great importance because it depends on the partial pressure of oxygen of the medium, and on the concentration of enzyme systems and metabolites which are found in the cell. Furthermore, it is an indication of the form in which chemical energy is used by the cell. In the amoeba, the oxidation-reduction potential of the cytoplasm is approximately  $-0.275$  volts in anaerobiosis and  $+0.070$  volts in the presence of oxygen

The basic cytoplasm appears to have a fundamental role in cellular metabolism although its functional modifications, owing to the lack of visible structure, are difficult to follow under the microscope. It is known that numerous cellular enzymes are located in the cytoplasmic matrix (see Chapter X). Thus, the peptidases (Holter), the oxidases and peroxidases are found chiefly in this part of the cytoplasm. Also here are found glutathione, vitamin C, ribonucleic acid, and so forth.

### The Nucleus

On vital or supravital examination, the nucleus appears as a more refractive sphere included in the midst of the cytoplasm and separated from this by the karyotheca or nuclear membrane (Fig. 12). Its interior is, in general, homogeneous except for the

presence of one or more refractive spherical bodies called nucleoli. This aspect is not, however constant. In some cases the nucleus has a granular aspect, the granules being more or less abundant but without visible connections. In a third group, one can distinguish nuclei in which the granules are united by lines forming a type of reticulum (Martens).

Under the ultramicroscope the nucleus appears optically empty but the nucleoli may be distinguished as luminous bodies (Fig 12). With micromanipulation it is demonstrated that some nuclei are denser than the cytoplasm and even can be extracted intact with the microneedle. In other cases, when the nuclear membrane is perforated, a liquid material flows out of the nucleus, the nuclear sap or karyolymph. Under the microneedle the nuclear membrane behaves like a true morphological membrane and not as a simple interface, for it opposes resistance to external pressure and can even become folded or wrinkled. Some authors consider it as a membrane of precipitation, produced in the nucleocytoplasmic interface.

When once the microneedle breaks through the membrane, it can be moved in the interior of the nucleus without encountering resistance and the nucleolus can be displaced with ease. The injury produced may provoke a localized gelation and even great changes which consist in the appearance of dense aggregates of material which are similar to the chromatic granules found in fixed material. Similar modifications are seen in cultured cells when they are submitted to the action of vapors of formol, acetic acid, ether and so forth. If these factors act only a short time, the changes may be reversible and the nucleus may recover its normal aspect. The problem of the existence of these structures in the living nucleus is of particular importance from the cytogenetic point of view in connection with the question of the continuity or lack of continuity of the chromosomes between two mitotic divisions (see Chapter VII).

### GENERAL MORPHOLOGY OF THE FIXED CELL

While the examination of living cells offers few morphological details, with methods of fixation and coloration the aspect of the cells is more complex and varied. Besides the structures that are observed *in vivo* which are more or less modified by fixation, others are found which were not apparent before owing to the similarity of their index of refraction with that of the rest of the cell.

The interpretation of these structural aspects should be made with caution, but not with excessive skepticism. Besides the ex

amination of living cells, the examination of fixed and colored cells is indispensable in order to learn more about cellular structure. The two methods do not exclude one another; they are mutually complementary.

It is interesting in this respect to recall what has been the historical evolution of technical methods of examination. Up until the middle of the nineteenth century observations were carried out almost exclusively on isolated cells, fine membranes, dissociated material or material pressed between two slides, and so forth. In contrast, in the second half of the century with the development of the methods of fixation, the techniques of embedding and sectioning and staining with different coloring materials, the interest of the investigators was transferred to dead cells, in which numerous details of structure were discovered. During this period, there appeared a series of theories which attempted to interpret the structure of protoplasm and, in general, of all the elements of the cell, on the basis of the aspect which the cell shows after fixation and staining. These theories today present only an historical interest. The principal ones are the *fibrillar theory* which considers protoplasm as constituted of fine fibrillae which run through a homogeneous basic material; the *reticular theory*, a variant of the preceding according to which the protoplasmic filaments are united into a vast network; the *alveolar theory*, according to which protoplasm is composed of a series of alveolar spheres pressed together (alveoli) so that they take an angular form, in the midst of a continuous phase, the hyaloplasm; the *granular theory* which affirms that in the midst of a homogeneous substance there exist granules, the "bioblasts" of Altmann.

Toward the end of the century a wave of skepticism spread among the investigators as a consequence of the work of Fischer and of Hardy. The former demonstrated that if different fixatives were made to act on homogeneous solutions of different proteins, such as albumose, gelatin, egg albumin, or peptone, there appeared filamentous, reticular, spongy and granular structures which correspond exactly to those observed in fixed cells. The aspect of the coagulation, which is produced by the action of the fixative, depends upon different factors, such as the nature and concentration of the fixative, the nature (molecular size, degree of hydrophilia) and concentration of the protein and the temperature of the environment.

For his part, Hardy found that in gels fixed under tension there appeared fibrillar figures, which orientated themselves following the lines of force, taking the aspect of asters, achromatic

spindles, and so forth, formations which appear in the cells in certain functional states. Thus, if with a platinum ring one supports a layer of gelatin and in the center of this deposits a small drop of mercury as the fixative acts there appear radial fibrillae which run out from the point where the pressure is exerted.

These investigations provoked a profound reaction. Some authors assumed a nihilist attitude with respect to the cellular structure and thought, unjustifiably, that all the details which appeared in the fixed preparations were artifacts of technique. In general, the reaction was, nevertheless, favorable and useful, because it put the cytologists on guard against the errors of technique and caused a revival of the study of living protoplasm. In recent years, it has been recognized that many of the so-called artifacts of technique actually exist in the cell, that others are no more than the manifestation of molecular or micellar changes produced by the action of the fixative on the submicroscopic structure of the cell (see Chapter IV) and that the true knowledge of the cellular structure results from the coordinated use of all of the methods of investigation.

## Fixation

Fixation is essentially a method of preservation of the morphology and the chemical composition of the cell. The object of fixation is to bring about the death of the cell in such a manner that the structure which the living cell possessed is conserved with the minimal addition of artifacts. Some methods, at the same time, attempt to maintain as intact as possible the chemical composition of the cell. Fixation has, then, two fundamental aspects, one, cytomorphological and the other cytochemical.

We shall not treat here in detail the methods of fixation employed in cytology. For this purpose, the treatises of histological technique of Romeis, McClung, Bensley and others should be consulted. We desire only to attempt a discussion of the problem of fixation insofar as it concerns the morphology and chemistry of the cell.

The majority of fixatives are aqueous liquids which act essentially upon the protein part of the cell. The most important conclusion of the works carried out on the colloidal models (Fischer, Hardy, Bütschli) is that in fixation there is a separation of the solid phase (dispersed phase) of the colloid from the liquid (dispersing phase) and that the former precipitates in the form of granules, nets, flakes, and so forth. In selecting a cytological

fixative one should seek that which precipitates the proteins in the finest form and, if possible, in ultramicroscopic aggregates, so that the aspect of the cell is not modified. Particularly valuable in this respect are some fixatives (formaldehyde) which act as polymerizing agents as well as precipitants.

When a piece of tissue is submerged in a fixing liquid, the death of the cells does not occur in an instantaneous manner. The fixative penetrates into the piece by diffusion from the periphery toward the center in such a manner that the most external cells are fixed more rapidly and better than the central ones. For this reason, in every fixed tissue, there is always a *gradient of fixation* which depends upon the *penetrability* of the fixative, its progressive *dilution* with the liquid of the cells, and the postmortem alterations which occur in the cells due to anoxia, changes in the concentration of hydrogen ions and enzymatic action (autolysis). The rapidity with which the fixative penetrates does not appear to depend so much on its coefficient of diffusibility as on the protein barrier of precipitation which is produced in the peripheral part of the piece. Thus, for example, with osmic acid the precipitation is very fine and a dense mass is formed which acts as a barrier impeding further passage of the fixative. For this reason fixation with osmic liquids is made with very thin pieces (0.5–1.0 mm).

Owing to the fact that the fixatives diffuse into the cells, currents are produced in the latter which frequently displace the soluble components. In Figure 15 one can see how an aqueous fixative modifies the distribution of cytoplasmic glycogen and its state of aggregation.

Besides displacing the soluble substances, fixatives *extract* with greater or less intensity. Thus, the electrolytes, soluble carbohydrates and even some lipids, may leave the cells by the action of the fixatives. It has been demonstrated that from 10 to 14 per cent of mineral substances of the cells are extracted by fixation (Pollicard and Okkels).

On the other hand, fixation and later treatment produce a *shrinkage* of the piece. This has importance since in interpreting the cytological images in fixed tissues one should always remember that the volume of the fixed cells is less than that which they had in the living state.

### Fixation by Freezing and Drying

From the analysis of the process of fixation which we have made, it follows that although fixation permits to some extent

the preservation of the cell in its true morphological aspect it may produce considerable chemical modification. Hence it was important to develop a procedure which would permit us to investigate the morphological structure and the distribution of the chemical components with a minimum of modifications. Such a technique was conceived at the end of the past century (Altmann, 1890) but was brought to practical use only recently (Gersh, 1932)

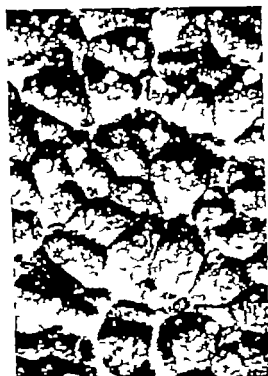


Fig 15

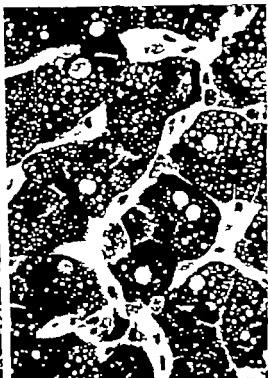


Fig 16

Fig. 15 Hepatic cells of *Amblystoma* fixed in Zenker formol. The diffusion current produced by the chemical fixative (from the lower to the upper part of the figure) displaces the glycogen of the cell. Stain. Best's carmalum. (From a preparation of I. Gersh.)

Fig. 16 Hepatic cells of *Amblystoma* fixed by freezing and drying. The glycogen appears distributed in homogeneous form in the cytoplasm. Nuclei and fatty droplets of spherical form can be distinguished. Stain. Best's carmalum. (From a preparation of I. Gersh.)

Drying can be accomplished by reducing the partial pressure of water vapor in the atmosphere surrounding the frozen tissue to a point below the vapor pressure of water at the drying temperature (Fig 17). When this is achieved at low drying temperature, the water passes off, from the solid phase in the frozen tissues, directly into a gaseous state (sublimation) without any intervening liquid phase which might distort the cell. In this way there is produced a progressive dehydration which reaches

practically to the extraction of all of the water contained in the tissues except for a residue which is tightly bound (For more details on this technique consult the works of Gersh, Scott, Simpson, Bensley De Robertis)

The advantages of this method are obvious. It does not produce shrinkage of the tissue the fixation is more or less homogeneous in the entire thickness of the piece there is not an extraction of soluble substances the chemical composition is

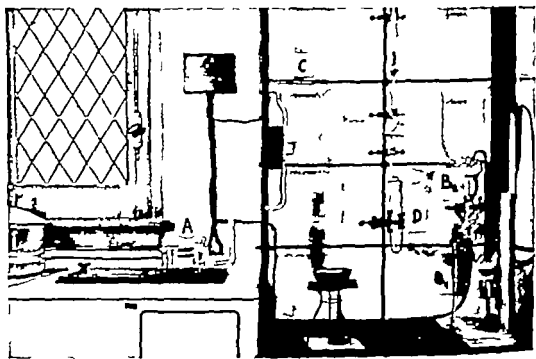


Fig 17 Freezing-drying apparatus installed in the Institute of General Anatomy and Embryology Buenos Aires. The frozen pieces are maintained at  $-30^{\circ}\text{C}$ . in the tube (A) submerged in a refrigerator. With a mechanical pump (whose tube of communication [B<sub>1</sub>] appears in the figure) and a diffusion pump (B<sub>2</sub>) a high vacuum is obtained. In the tube (C) a hygroscopic substance (phosphorus pentoxide) is placed which retains the water extracted from the tissues. The tube (D) permits carrying out of paraffin embedding in the vacuum.

maintained practically without change, and the structure, in general, is preserved, with very few modifications produced by the ice crystals (Fig. 16). Furthermore, with this method, the cessation of the vital phenomena is almost instantaneous and there is not time for the production of "postmortem" chemical alterations. This rapidity of fixation permits one to trap and preserve cells at critical moments of their function such as at the moment when kidney cells are excreting colored material or other substances (Gersh) or when thyroid cells are extruding colloid droplets into the follicular cavity (Fig. 129).

The technique of freezing-drying of Altmann Gersh should be considered as an intermediary between the examination of fresh and fixed tissues. We are not treating of a true fixation in the strict sense, because it lacks the characteristic of complete irreversibility. Many of the cellular components are preserved in the same soluble form as in the living state. Thus the solubility of many proteins, glycogen, salts, and so forth, is preserved, a fact which permits one to study the action of different solvents on the structure and composition of the cell, with the advantage that the action of them is not limited by the semipermeable barriers which are formed by the cell membranes of living cells. In addition, certain enzyme systems are preserved which can continue acting when the liquid phase is added (proteolytic enzymes, glycogenolytic enzymes, and so forth). Recently it has been possible to demonstrate that frozen and dried liver cells maintain the power to consume oxygen, although to a very reduced extent (see Chapter X).

### Structure of the Fixed Cell

In the fixed cell, the same fundamental elements are distinguished (cytosome, nucleus and membrane) as in the living. In the cytosome, as well as in the nucleus, a series of formations can be distinguished, some of which are visible in the immediate examination, but others are apparent only after an adequate fixation and staining (Fig. 18).

In the cytosome, the most fundamental part, the *cytoplasmic matrix* appears in the form of a precipitate, more or less fine according to the fixative used. With appropriate methods, one distinguishes further a *formed part* composed of a series of different formations, which are classified as *organoids* and *inclusions* according to the constancy with which they are present and the importance which they have in the physiology of the cell.

The organoids are found in practically all cells. To them has been attributed the property of dividing and of perpetuating themselves and it is believed that they play an important role in the life of the cell. In animal cells they include the *chondriome*, the *Golgi apparatus* and the *cell center*. In plant cells are found, in addition, the *plastids* which play a very important role in metabolism.

These organoids will be studied extensively in Chapter V. The *chondriome* is constituted by the whole number of *chondriosomes*, bodies of filamentous or granular form, visible in the fresh condition (Fig. 12) and which can be fixed by special methods. The

Golgi apparatus generally is seen as an irregular reticulum situated around the nucleus (Fig 42) or in a localized part of the cell. It is invisible in the majority of living cells, but can be demonstrated by osmic and silver impregnation. The *cell center* is an organoid related to cellular division which is composed of one or two little bodies (*centrioles*) in the midst of a spherical mass, the microcentrum. During division the cell center reaches its maximal development. At the same time there appears about the microcentrum a clear gel like zone (*centrosphere*) from which there extend radiations (*astrosphere*)

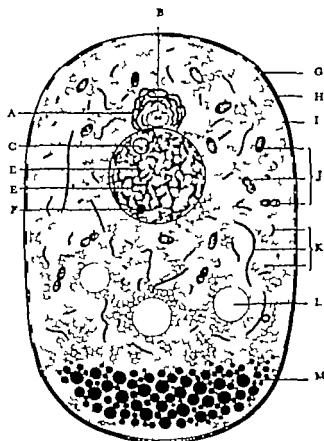


Fig. 18 Diagram of an animal cell: *A* Golgi apparatus; *B* cell center *C* nucleolus; *E*, chromonema; *F* karyosome or false nucleolus; *G* cell wall (pellicle) *H* plasma membrane (invisible); *I* cortical cytoplasm; *J* plastids; *K* chondriosomes, *L*, vacuole; *M* cellular inclusions (drops of fat, and so forth) (From E. B. Wilson.)

In some specialized cells (nerve cells, gland cells) there exists a substance disposed in the cytoplasmic matrix in a diffuse or fibrillar form, or in large or small flakes. This has special staining properties which depend on certain chemical characteristics. It is called the *chromophile chromidial substance* or the *ergastoplasm* which, according to some authors, should be considered as a special part of the basic cytoplasm.

In the differentiated cells one may encounter likewise a series of *fibrillar* formations, such as the *tonofibrillae* *neurofibrillae* and *myofibrillae* and *cilia*, which are the product of a functional adaptation (differentiation) of the cytoplasm

The *inclusions* or *paraplasts* comprise accumulations of material which are not constant but which are considered to be either ingested substances or as products elaborated by the cell, or resulting from the destruction of substances (catabolism) on the part of the cell. Such inclusions are the *drops of fat* the *carbohydrates granules of protein, pigment crystals* and *secretion granules* (Fig 18)

In the *fixed nucleus* one can distinguish (1) the limiting *nuclear membrane* (2) a clear mass, the *nuclear sap* or *karyolymph*, which fills completely the nucleus and in which are found included the other components (3) a series of twisted and interlaced filaments, the *chromonemata*, which contain a substance, *chromatin*, which stains intensely with certain coloring matters (frequently the chromonemata appear connected together by fine filaments—the so-called *linin*—which seem to form a reticulum) (4) in some nuclei there are found larger and denser flakes of chromatin situated in the chromonemata, the *chromocenters* or *karyosomes* also called *false nucleoli* or *chromatin nucleoli* (5) one or more spherical bodies, the *nucleoli* which differ from the chromocenters in their chemical composition and which can be distinguished with appropriate methods of staining (Fig 53)

The *cell membrane* comprises the *plasma membrane* generally invisible, and the external protective layers

Figure 18 is a diagram in which are represented the different formations which can be seen in the cells when one utilizes a series of methods of fixation and coloration

In Chapters V VI, VII and XII we shall study in detail the *organoids inclusions* and *differentiations* of the cytoplasm, the *cell membrane* and the *morphology* and *chemical composition* of the nucleus.

## Form and Size of the Cell

There exist cells which have a *variable form* such as the amebae and leucocytes, and cells with a *stable form*, such as the spermatozooids, infusoria, erythrocytes, epithelial cells and nerve cells. These latter have in every case a characteristic relatively fixed form which represents, in general, a specific character of the cell type.

The form of the cell depends in part upon the surface tension and viscosity of the protoplast, the mechanical action which the adjoining cells exert, the rigidity of the membrane and the functional adaptation. Many cells when isolated in a liquid medium tend to take a spherical form, obeying the laws of surface tension. This is the case with the leucocytes, which in the circulating blood are spherical, but which by the influence of adequate stimuli can emit pseudopods (ameboid movement) and become completely irregular in shape.

The cells of many plant and animal tissues have a polyhedral form, statistically more or less constant, which is determined principally by reciprocal pressures. In these cases, the original spherical form is modified by contact with the other cells, just as in the foam from soap each bubble is pressed by its neighbors.

Individual cells in a large mass appear to behave like polyhedral solids of minimal surface packed without interstices. Although regular polyhedra of 4, 6 and 12 sides can be packed without interstices, the 14 sided polyhedron (or tetrakaidecahedron) satisfies most closely the conditions of minimal surface. The study of soap bubbles in foam by Plateau showed that these conditions apply in such a system, and that the average bubble had fourteen sides. It was later demonstrated that minimal surface of packed tetrakaidecahedra was achieved, not by solids with planar faces (orthic polyhedra) but by solids with eight hexagonal nonplanar faces and six quadrilateral surfaces with curved edges (Lord Kelvin). This ideal form is rarely encountered in cells, but reconstructions and countings of surfaces carried out on a considerable number of different animal and plant cells in appropriate masses (Lewis) revealed an average figure very close to that of fourteen faces.

The *volume* of the cell is variable and oscillates within broad limits. In plants and in animals, cells are found which are visible to the naked eye and which possess a very great volume. Thus, the eggs of certain birds may have a diameter of several centimeters and are composed, at least at first, of a single cell. This is, nevertheless, the exception, the great majority of cells being visible only with the microscope, their diameter measuring some few thousandths of a millimeter (micra,  $\mu$ ). The smallest cells have a diameter of four micra (four one-thousands of a millimeter). In the tissues of the human body, if one excepts the nerve cells, the volume varies between  $200 \mu^3$  and  $15,000 \mu^3$  (Levi). In general, the volume of the cell is fairly constant for any one cell type and independent of the size of the individual. For example, the renal or hepatic cells of a bull, of a horse, or of

a mouse have an almost equal size. The differences in the total mass of the organ are due to the number and not to the volume of the cells (*Driesch's law of constant cellular volume*)

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## Chapter IV

### SUBMICROSCOPIC ORGANIZATION OF THE CELL

In the two preceding chapters we studied living matter or protoplasm from the point of view of its chemical and physico-chemical composition and of its morphological organization. In the second chapter it was shown that living matter is composed essentially of *water organic substances* (proteins, lipids, and carbohydrates) and *inorganic substances* (mineral salts) dissolved in the liquid phase or in a colloidal state. In the third chapter it was explained that living matter is subdivided into minute units, the cells, in which one distinguishes under the microscope a series of components (cytosome, nucleus, membrane) and different particles (mitochondria, inclusions, chromonemata, nucleoli, and so on)

These conclusions are the result of investigations employing various methods, including qualitative and quantitative biochemical analysis in one case and microscopic examination in the other applied to elements of very different dimensions (molecules on the one hand and cells on the other) and, therefore, belonging to two distinct levels of organization

Biochemical analysis has as a point of departure the destruction of all of the cellular structure. The tissues are triturated, extracted with different solvents, digested, and so forth, until one has separated and obtained in an analyzable state their fundamental components. From such analyses, one can learn something of the elemental molecular units, but ordinarily one cannot determine the form in which the units are associated and grouped to constitute the edifice of the cell. On the other hand, cytological analysis is limited by the resolving power of the microscope which is very far above molecular dimensions (see below)

In recent years, the construction of a bridge between these two levels of knowledge has been initiated. This has been due to the adaptation and the employment in biology of techniques derived from physics and chemistry and to the breaking down of the barriers which previously separated these sciences. Below the structure visible to the microscope there exists a true organization of molecules and micelles in the different phases of the system which constitute protoplasm.

The knowledge of this *submicroscopic structure* or *ultrastructure* is of fundamental importance, since all the essential physicochemical transformations which characterize vital phenomena take place at this level. Such transformations can, at times, have a cytological expression, as for example, in the case of cellular division, in which case the entire cell is modified, or in that of the changes which occur in the Golgi apparatus and in the chondriome during the secretory cycle. In other cases, as in many metabolic processes, there are no visible modifications of the microscopic structures, because those processes take place exclusively in the realm of the ultrastructure. Thus, for example, the phenomena of cellular respiration or the penetration of ions or molecules across the plasma membrane may give no manifestation visible to the microscope, but occur amidst the molecular architecture of the cell. It may thus be understood that a cell may undergo various functional and even pathological changes without the occurrence of any cytological manifestation visible to the microscope. While strictly speaking the determination of tissue ultrastructure is a branch of *morphology* it is intimately connected with *chemistry* since it is essentially the morphology of the molecular complexes. It is, furthermore, a subject of great interest also to physiologists, for just as a knowledge of gross and microscopic anatomy was essential to the development of *physiology* in the past, so in the modern era with its great advances in the structural chemistry of complex biological substances, a knowledge of cellular and tissue ultrastructure is fundamental in order to discover the mechanisms which underlie tissue and cell function (Schmitt). Likewise it is important for *embryology* because many morphogenetic phenomena may be based on modifications of the ultrastructure, and for *genetics* which has as one of its aims the explanation of the mechanism of chromosome division and the ascertaining of the molecular structure of the chromosome and of the genes.

### Limits and Dimensions

To interpret better the results of submicroscopic analysis and the basis of the methods employed it is necessary to note clearly the limits which separate the two levels of organization and the dimensions of the units which compose them. We have seen that the cellular structures are measured in *micra* ( $\mu$ ). The ultramicroscopic particles have dimensions very much smaller which makes convenient the utilization of other units of measurement. These are the millimicron (m $\mu$ ) which corresponds to the thousandth part of a micron, and the *Angström unit* (Å) used particularly to

measure the wavelengths of radiations, which corresponds to 0.1 of a  $\mu$ , that is to say one ten thousandth of a micron (Table IV Chapter II)



Fig. 19 Comparison between the dimensions of molecules and of cells with the wavelengths of various radiations. (From A. B. Hastings, T. Young and J. B. Hoag.)

In Figure 19 the dimensions of different cells, bacteria, viruses and molecules are indicated on a logarithmic scale. Furthermore, these dimensions are compared with the wavelength of various radiations. The limit of microscopic vision depends directly upon the wavelength of the visible radiations of the solar spectrum which go from about 7500 Å in the extreme red to about 3900 Å in the violet. In actual practice, one can distinguish particles which have a diameter as small as 0.25  $\mu$ , that is to say 2500 Å, although the limit of definition in certain conditions is somewhat below this figure (see page 68). In general, all the cytological structures, mitochondria, centrioles, nucleoli and so forth, have dimensions larger than 0.25  $\mu$ , but there are also observed particles which approach the limits of microscopic resolution.

Most molecular dimensions are, on the other hand, very far below these limits. Thus, a molecule of glucose has a diameter of only 5 Å, so that one billion of these molecules would be necessary to make up the smallest particle which attains the limits of microscopic vision, and to form a mitochondrion there would be necessary one million particles of the size of a protein molecule of 100 Å.

To summarize we can say that the resolving power of the compound microscope with visible light marks the limit between the microscopic and the submicroscopic, or between the microscopic structure and the ultrastructure.

Investigation of the ultrastructure requires special methods which we shall describe below.

## Methods

The methods for the analysis of the ultrastructure may be classified into *direct* and *indirect*. Among the *direct* are the ultra

violet microscope, the fluorescence microscope and, especially the electron microscope

*Ultraviolet Microscope* This microscope differs from the ordinary microscope by the employment of ultraviolet radiations and by possessing an optical system of quartz or other dielectric substances transmitting in the ultraviolet. In the case of objectives corrected to a wavelength of 2750 Å, the power of resolution is only doubled (see below) Hence, this method offers such a slight gain in resolution

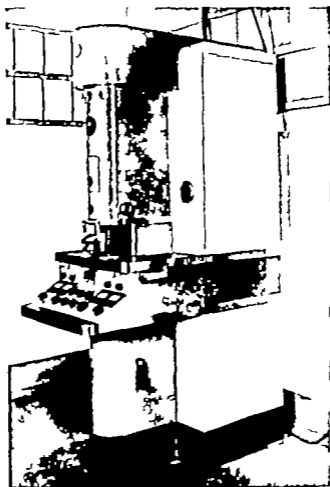


Fig 20 R.C.A. Universal model electron microscope (E.M.U.) (Courtesy of R. C. A.)

that it has not proved to be valuable on this account. On the other hand, if a monochromator is adapted to the microscope so as to permit the study of the absorption or the effect of different monochromatic portions of the spectrum, the method acquires a great value in histochemical study for with it the cellular localization of nucleic acid and of other substances is possible (see Chapter VII). Likewise, the fluorescence microscope has limited application in the analysis of the ultrastructure. In this instrument ultraviolet light is used, and use is made of the fluorescence which certain structures emit when they are irradiated. This method is important in determining the localization of vitamin A, thiamine, riboflavin and of other fluorescent substances in the cell. The property of fluorescence can be induced in many structures with the use of fluorescent substances. Recently this method has been used for the histochemical localization of sulfonamides. The histochemical value of this method has been increased considerably with the spectrographic analysis of the radiation emitted by various substances (Sjostrand)

**Electron Microscope** This is the only instrument which permits a direct study of the ultrastructure, since its power of resolution is much greater than that of the light microscope or of the ultraviolet microscope (Fig 20) This instrument utilizes the property possessed by streams of electrons of being deflected from their course by an electrostatic or electromagnetic field, in

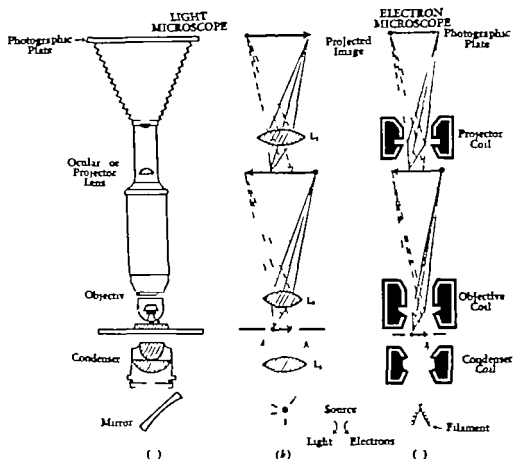


Fig. 21 Comparison between the optical microscope and the electron microscope. (From G. Thompson.)

the same way that a beam of light is refracted on crossing a lens. If a metal filament be placed in a highly evacuated tube, when heated it emits electrons that can be accelerated by means of a difference of electrical potential. This stream of electrons tends to follow a straight path and has properties similar to those of light and, like light, manifests a corpuscular and vibratory character at the same time, but has a very small wavelength.

The construction of an electron microscope utilizes elements analogous to those in the optical microscope (Fig 21) In this case, the source of 'light' is the filament of the cathode which emits the stream of electrons. By means of a magnetic coil, acting

as a condenser the stream of electrons is focused in the plane where the object is located. After traversing this object, the stream of electrons is deflected by another magnetic coil which acts as an objective lens and gives a magnified image of the object. This is received by a third magnetic lens, which acts as an ocular or projection lens and magnifies the image from the objective. The final image can be visualized on a fluorescent screen or recorded on a photographic plate.

In Figure 21, one can follow the path of the electrons and the formation of the images by the objective and by the projection lens of the electron microscope, and at the same time one can appreciate the similarity which this instrument has to the optical microscope.

*The power of resolution or of definition of the electron microscope is determined by the same variants as in the light microscope. In any microscope the power of resolution, which is the capacity to give distinct images from points very close together in the object, depends upon the wavelength ( $\lambda$ ) and upon the numerical aperture (A) of the objective. The limit of resolution, which is the minimum distance between two points in order that they may be defined, is*

$$\text{Lim Res.} = \frac{0.6\lambda}{A} \quad (1)$$

The numerical aperture being:

$$A = n \sin \alpha \quad (2)$$

(where  $n$  is the index of refraction of the medium and  $\sin \alpha$  the sine of the semi-angle of the aperture)

In the case of the light microscope even if one uses monochromatic light ( $\lambda = 4,000 \text{ \AA}$ ) and lenses of the maximum numerical aperture attainable (1.43) one arrives at a limit of resolution of approximately  $1700 \text{ \AA}$  ( $0.17 \mu$ ). In practice with white light the resolving power of the microscope is less (about  $2500 \text{ \AA}$ ).

From formula (1) one can deduce that to increase the power of definition, the numerical aperture (A) being fixed, it is necessary to diminish the wavelength ( $\lambda$ ). This is accomplished by the ultraviolet microscope, and very much more effectively by the electron microscope.

The wavelength of a stream of electrons is a function of the acceleration voltage (V) or potential to which the electrons are subjected, and can be calculated in an approximate form by the formula of De Broglie

$$\lambda = \frac{12.3 \text{ \AA}}{\sqrt{V}} \quad (3)$$

For example, in one of the standard current models, the RCA EMU electron microscope,  $V = 50,000$  volts and  $\lambda = 0.0535 \text{ \AA}$ . With this wavelength and with a numerical aperture of 1.40 as in the optical microscope (L) the resolution would be increased by 80,000 times.

Nevertheless, due to the great aberration of the magnetic lenses, the actual numerical aperture of the electron microscope is very small and the limit of resolution less. This may be calculated theoretically as between 6 and 10  $\text{\AA}$ , at

though the majority of published photomicrographs show a resolution of 50 to 100 Å. Still in some special cases, it has been possible to arrive at a resolution of 20 Å and even less" (Hall)

In the optical microscope the magnification is determined largely by the objective, which reaches a maximum of 100 to 120 magnifications. The ocular can increase this image some 5 to 15 times. A total useful magnification of  $500\times$  to  $1500\times$  can be achieved. In the electron microscope the resolving power is so high that the image from the objective can be enlarged very much more. Thus, with an initial magnification by the objective of  $100\times$  the image can be magnified 200 times with the projector coil, the total magnification thus being  $20\,000\times$ . In some cases, the total magnification can be profitably increased to 100 000 diameters by photographic enlargement.

By its extraordinary resolving power the electron microscope seems to be an ideal instrument for the study of the submicroscopic structure of the cell. Nevertheless, its usefulness is reduced by a number of technical difficulties and of limitations which may in time be partly overcome.

One limitation is due to the low power of penetration of the electrons. The specimen to be examined must be very thin. It is generally deposited on an extremely fine layer of collodion (75 to 150 Å in thickness) which serves as an object holder and is supported by a fine metal grid. If the thickness of the specimen exceeds 5 000 Å ( $0.5\ \mu$ ) it appears almost totally opaque.

Another limitation comes from the fact that, since the specimen must be placed in a high vacuum, it must be dehydrated. This prohibits the study of cells in the living state.

On the other hand, the structures which the electron microscope reveals directly are only the manifestation of differences in thickness or in electron density (Fig 45). Recently however attempts have been made to use the electron beam indirectly for the analysis of elements in chemical compounds (microanalyzer of Hilber).

Recently important advances have been made in techniques of electron microscopy of biological materials. The use of evaporated metals (shadow-casting) on specimens has led to a considerable increase in the contrast and definition of images and of finer details of the surface of objects (Williams and Wyckoff). This technique consists in placing the specimen in an evacuated chamber and in evaporating a heavy metal such as gold or chromium from a filament of incandescent tungsten. The grid is placed at a small angle to a straight line from the filament, whereupon the deposit of material is made on one side of the

surface of the elevated particles and on the other there is formed a shadow, the length of which allows us to ascertain the height of the particle. The photomicrographs made of such specimens have a three-dimensional aspect which is normally lacking (Fig 1)

An increase in contrast of the submicroscopic structures has been recently obtained by the use of substances which contain heavy atoms such as osmic acid and phosphotungstic acid, which have the property of scattering electrons. These substances may under certain conditions act as electron stains, comparable to the histological stains, combining selectively with certain regions in the specimen. The use of electron stains has given excellent results in the visualization of the fine ultrastructure of collagen fibers and muscle fibers (Figs 30 and 136)

The difficulty which results from the thickness of cells has been surmounted up to a certain point by the use of special microtomes in which the blade is mounted on a wheel which runs at 25 000 r p m. while the specimen is advanced at a constant rate. Under these conditions, sections 0.1  $\mu$  in thickness have been reported. In such sections, portions of the cells are penetrable by the electron beam, and photographs of the specimen have been obtained (Gessler and Fullam)

Another promising technique consists in making a culture of tissue on a coverslip covered with a layer of collodion. The culture can be removed along with the collodion and observed with the electron microscope. As the cells flatten out considerably as they spread over the smooth surfaces, parts of the cytoplasm become sufficiently thin to be penetrable by the beam of electrons and show various details of their structure and ultrastructure (Fig 22) (Porter Claude and Fullam)

Among the *indirect methods* of determining ultrastructure the most important are the use of polarization microscopy and of x ray diffraction.

**Polarization Microscopy** This method, which is the oldest of those utilized in the study of ultrastructure, is based on the behavior which certain components of cells and tissues have when they are observed with polarized light. If the material is *isotropic*, light is propagated through the material with the same velocity whatever may be the direction of the plane of the polarization of light. Such substances or structures are characterized by having the same *index of refraction* in all planes. On the other hand, in an *anisotropic* material the velocity of propagation of polarized light varies according to the direction of propagation with respect to some axis of the substance. Such a material is said to be

birefringent because it presents two different indices of refraction corresponding to the respective different velocities of transmission.

The birefringence may be expressed quantitatively as the dif



Fig 22. Electron micrograph of a portion of a fibroblast like cell cultured from chick embryo tissue on a formvar film. Fixation in osmium tetroxide vapors. Nucleus in the upper left angle. Filamentous chondriosomes show zones of different density. Granular structure in the ground cytoplasm.  $\times 3,600$  (Courtesy of K. R. Porter, A. Claude and E. F. Fullam and of *Radiography and Clinical Photography* 22 1946)

ference between the two indices of refraction ( $N - N_0$ ). In practice, one measures the retardation ( $\Gamma$ ) [in  $m\mu$  or in fractions of wavelength ( $\lambda$ )] which the light polarized in one plane experiences with respect to that in another plane perpendicular to it.

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The retardation depends on the thickness of the specimen ( $d$ ) in this manner:

$$\text{Birefringence (B)} = \Delta n = \frac{1}{d}$$

The measurement of the retardation is generally carried out by the use of some sort of compensator which can be introduced into the optical system.

The birefringence of biological materials is generally very small. Measurement of small retardations requires very sensitive compensators such as the Köhler compensator.

Let us consider now the situation which prevails in the majority of animal fibers, which can be thought of as bundles of anisotropic cylinders. In such cases if the direction of propagation of the polarized light is parallel to the long axis of the cylinder (which corresponds to the optic axis) birefringence is not apparent because the velocity of propagation of the beam is equal for all planes of polarization, which are radial to the fiber. But if the direction of propagation is perpendicular to the axis of the cylinder then the velocity of propagation will vary as the plane of polarization is rotated. It is said in such cases that the birefringence is *uniaxial*. It may be further designated as *positive* if the index of refraction along the length of the fiber is greater than in the perpendicular plane and *negative* in the opposite case. This characteristic is of particular importance to determine the nature of the substance which produces the birefringence.

The first application of the polarizing microscope to the study of structures was carried out on plant cells by Nägeli in the middle of the past century. As a consequence of these studies he formulated the *micellar theory* which has had important influence on our modern concepts of ultrastructure. Nägeli suggested the existence of ultramicroscopic particles which behave toward polarized light like minute crystals, exhibiting the phenomenon which later was called *crystalline birefringence* or *intrinsic birefringence*. Other types of birefringence found in biological materials are form birefringence, strain birefringence, rotary birefringence and dichroism.

*Crystalline* or *intrinsic birefringence* is found in systems where the bonds of molecules or ions have a regular asymmetrical arrangement, and is characterized by being independent of the index of refraction of the medium. In animal cells, in structures composed of proteins or of lipids, there may appear a certain degree of crystalline birefringence, which in both cases is positive uniaxial. On the other hand, the fibers of nucleoprotein do not

follow this rule, as their content of nucleic acid endows them with *negative uniaxial* birefringence

In structures (fibers or membranes) formed by the association of lipids and proteins the chainlike molecules of the lipids are in general oriented with their long axes in a perpendicular direction with respect to the protein polypeptide chains (Fig 23). In this case, each of the respective structural members tends to give opposing signs of birefringence (see in Chapter VI the molecular structure of the plasma membrane). If a fiber has negative birefringence with respect to its long axis, one may suspect that it may be constituted of a lipoprotein with lipid molecules orientated perpendicularly or of nucleoproteins. In such a case one can apply fat solvents and if the negative birefringence is decreased one has evidence of a lipid component in the fiber.

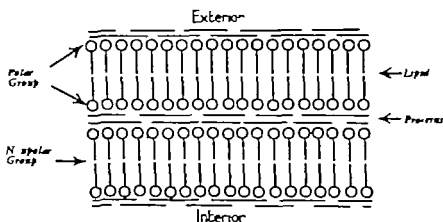


Fig 23. Molecular structure of a lipoprotein membrane (myelin sheath)

*Form birefringence* is produced when submicroscopic asymmetrical particles are found orientated in a medium of a different index of refraction. In this case, the birefringence is changed when the index of refraction of the medium is changed and it can be abolished, except for *intrinsic birefringence*, when the refractive index of the medium is equal to that of the particles.

According to the Wiener theory if the particles are cylinders oriented with their long axes parallel to the axis of the fiber the birefringence is positive; if they are platelets, with their axes oriented perpendicularly the birefringence is negative with respect to the axis of the fiber. By the immersion of a structure in media of different indices of refraction and measuring the birefringence in each case, one can construct curves in which birefringence is plotted against index of refraction. The position of the minimum of these curves indicates the presence or absence of form birefringence, and also tells if the form birefringence is

pure or as is generally the case, if it is combined in greater or less degree with crystalline birefringence (Fig 24) (For more details on the technique, see the works of W J Schmidt, 1924 1937 Frey Wyssling 1938 Schmitt, 1944, Bennett, 1948)

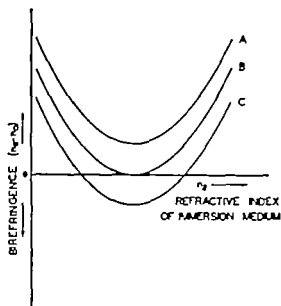


Fig. 24 Method of determining sign and relative amount of form and crystal line birefringence by immersion technique. A indicates positive form and positive crystalline birefringence. B indicates positive form and no crystalline birefringence. C indicates positive form and negative crystalline birefringence (From Schmitt.)

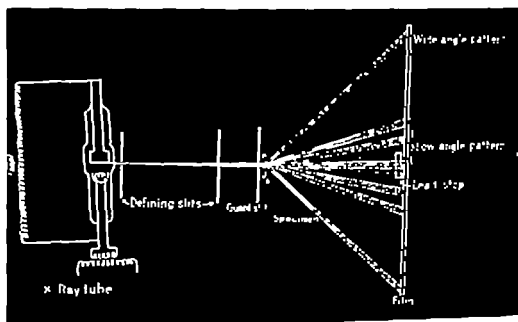


Fig. 25 Diagram of the x ray diffraction set-up for biological specimens. Two defining slits collimate a fine beam of x rays. The specimen is put behind the guard slit and the film at a distance varying between 3 to 30 cm. A lead plate stops the undiffracted beam. The position of the wide-angle and small-angle pattern on the film is indicated. Compare with Fig. 26. (Courtesy of R. S. Bear and O. Bolduan.)

*X ray Diffraction* This technique is based on the property which radiations have of being diffracted when they encounter small obstacles. If a ray of white light ( $\lambda$  averaging  $0.5 \mu$ ) impinges upon a diffraction grid which has one thousand lines per millimeter it will be diffracted, giving the various bands of the spectrum. But if the same grid is traversed by  $x$  rays (the wavelength of which is much smaller) no measurable diffraction will be produced. Laue suggested that grids of much smaller dimen-



Fig. 26 X ray diffraction pattern of beef tendon Fiber. *E* is vertical wide angle pattern. *E*, equatorial "side chain" spacings of  $11 \text{ \AA}$ . *M* meridional arch at about  $2.86 \text{ \AA}$ . *L*, meridional long spacings. Small angle pattern (in the lower left angle of the figure). The fundamental repeating period is of  $640 \text{ \AA}$ . (Courtesy of R. S. Bear.)

sions, such as are found in a crystal, would be necessary for the diffraction of  $x$  rays, and utilized crystals for this purpose. The atoms, ions, or molecules in crystals constitute a true lattice of molecular dimensions capable of diffracting radiations of this wavelength.

In essence, the technique consists in making a beam of collimated  $x$  rays traverse the material which is to be analyzed (nerve, hair, cellulose, and so forth) and in placing beyond this a photographic plate which records the diffraction pattern. On this there may appear a series of rings and concentric spots or

bands (Fig 26) The distance between the dark zones and the center depends upon the spacing which exists between regularly repeating units in the specimen which produces the diffraction (periods of identity) The less the angle of diffraction, the greater the distance between the repeating units (Fig 26)

The distance  $d$  between the periods of identity can be calculated by means of the equation known as Bragg's Law

$$n\lambda = 2d \sin \theta$$

$n$  = number of the diffraction order  
 $\lambda$  = wavelength,  
 $\theta$  = angle of incidence)

which shows that with a knowledge of the angle of incidence of a definite spot in the diffraction pattern and of the wavelength, the distance between the spacing producing the diffraction can be calculated. This relation is true only for crystals, not for liquids and gases. A wavelength frequently used is the  $K\alpha$  line of Cu (1.54 Å)

The degree of orientation of the particles is shown by the nature of the interference patterns. When the particles are unorientated, concentric circles are found. Orientation in fibers generally gives arcs or sickles, while a perfect orientation (as in crystals) is indicated by spots. In general, in orientated protein chains, such as characterize many biological fibers, the equatorial points have been considered to indicate the lateral separation between the individual chains, while the meridional points are thought, in certain cases, to represent the distance between the amino acid residues (Figs. 26 and 27)

As was indicated above, the distance between the spots or bands and the center of the diffraction pattern is smaller for diffraction effects due to longer spacings in the material. These long spacings, which may reach from 25 Å up to 1,000 Å in high polymers, are of great importance in biological materials. In wide-angle patterns (Figs. 25, 26) they appear as small meridional spots close to the center and thus are not clearly resolved. This difficulty has been reduced by using special technical refinements which permit one to obtain small-angle patterns and thus a much better resolution of the long spacings (Fig. 25). Later in this chapter and in Chapter XII, we shall mention the important results obtained by means of this technique in the study of the ultrastructure of collagenous fibers and of the muscle fibrillae.

X-ray diffraction is one of the most important methods for the study of ultrastructure in those cases where it is applicable, for it permits us not only to determine the orientation of the mole-

cules, but also to measure with exactness the distances which separate them and even to recognize, in part, the intramolecular organization.

Other indirect methods of less biological importance are electron diffraction, interference microscopy and darkfield microscopy (see Chapter II)

One of the most recently developed instruments for the study of the ultrastructure of membranes is the analytical leptoscope. This technique and its results will be discussed in Chapter VI

### Linear and Laminar Structure

The submicroscopic structures which are observed in biological materials by the methods cited above can be classified

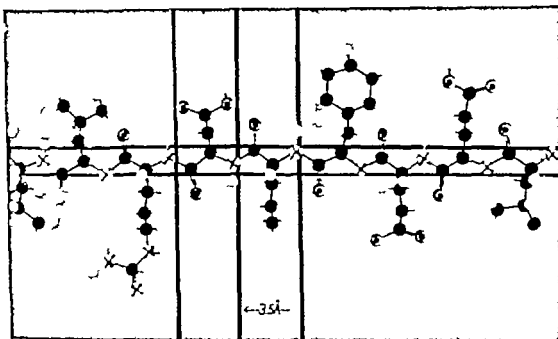
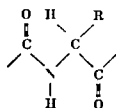


Fig. 27 Model of a fragment of a polypeptide chain made to scale, showing eight amino acid residues. The peptide chain and two residues are bordered by coarse lines. The black spheres represent carbon atoms, those with a central point, oxygen atoms and those with a cross, nitrogen atoms. The hydrogen atoms are indicated by small white spheres. (Courtesy of Spenser)

into two groups (Herzog). In one group are linear macromolecules, true chains of molecules which are associated in a crystal line form or as submicroscopic fibrillae. In the other group there are laminar structures formed by the association of "fibrillae" in one plane. These fibrillae and submicroscopic membranes can, at times, aggregate to form larger types of organization, visible to the microscope and even to the naked eye (cellular fibers and supracellular fibers of Picken)

In animal tissues there is a series of structures which are of this type of organization and which, according to Picken, can be classified into three categories, as follows *subcellular*, which comprise parts of cells, such as membranes, cilia and spindles *extracellular*, such as collagenous and elastic fibers, membranes of cellulose or chitin, which are situated outside of the cells and *supracellular* which are macroscopic structures, such as the hair bone and muscle, in which there exists a similar micellar and molecular organization.

In all these types of structures, especially in the animal fibers, proteins comprise the fundamental component. The *proteins* are composed of long chains of amino acids with peptide linkages and may or may not be united to other groups such as nucleic acid or carbohydrates. As may be noted in Figure 27, the polypeptide chain is composed of  $\alpha$  amino acids bonded by acid amide linkages, the side chains R projecting laterally. The fibrillar



structures are formed by the association of many of these long peptide chains parallel to each other and, in many cases, probably bound together laterally by means of side chain linkages.

The nature of the side chains and the degree of union which exists between the peptide chains influence the physicochemical properties of these fibers. Thus, for example, in fibers having an essentially mechanical function such as those of hair the protein chains are very closely pressed together the lateral unions are very strong and there is very little space to contain water. On the other hand, in tendon and other connective tissues, the lateral union is less strong, there is a greater space between the chains and the water content is much greater. These two contrasting types of fibers are called, respectively *keratinoid* and *collagenous*.

Astbury has postulated on the basis of x ray diffraction studies that the polypeptide chains which compose the proteins may have different configurations. Thus in the case of keratin of hair he distinguishes a *folded type* ( $\alpha$  keratin) and another *straight type* ( $\beta$  keratin) (Fig. 28). He explains many of the mechanical properties of these fibers on the basis of a transformation of one type of fiber into the other. In hair there is found, in general, the folded molecular configuration ( $\alpha$  keratin) but under the influence of tension or by the action of water vapor the hair elongates because of the straightening out of the protein chains ( $\beta$  keratin).

In a third type, the *corpuscular* Astbury postulates the peptide chain to be completely folded in the form of a molecular corpuscle (for example, in egg albumin, hemoglobin, and so forth) (Fig. 28)

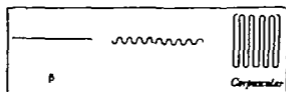


Fig. 28. Diagram representing the disposition of the polypeptide chain in a straight ( $\beta$ ) folded ( $\alpha$ ) and corpuscular protein, according to Astbury

In recent years, there has been a considerable advance in our knowledge of the ultrastructure of collagenous fibers. The electron microscope revealed that along the length of the collagenous fibrillae there are alternating dark and light bands, and that the distance between bands of equal density is about 644 Å (Fig. 29) (Schmitt, Hall and Jakus). Recently also by

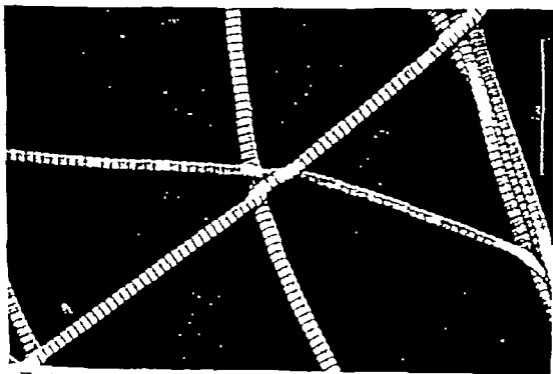


Fig. 29. Electron micrograph of collagen fibrils from human skin, shadowed with chromium. Bands with period of 640 Å are seen.  $\times 28,000$ . (Courtesy of Dr. J. Gross.)

use of phosphotungstic acid, it has been shown that within this period of 644 Å there is a series of at least five periodic bands which combine preferentially with the electron stain (Fig. 30). These results confirm those obtained earlier with the use of x ray diffraction (Bear). The characteristics which are most outstand

ing in the wide-angle x ray diffraction pattern of the collagenous tissues are the equatorial spots which indicate a lateral separation between the polypeptide chains of 11 Å and the meridional arc of 286 Å which, according to Astbury, indicates the distance between the residues of the amino acids along the chain (Fig 26). The use of the small-angle technique has permitted the determination of the presence of a fundamental spacing which is repeated regularly at intervals of 640 Å along the fiber (Bear Fig 26). This coincides with the evidence from the electron micro-

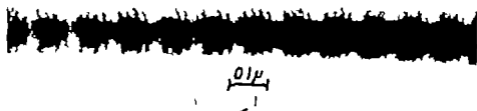


Fig. 30 Electron micrograph of a collagen fibrilla from rat tail tendon. Stained with phosphotungstic acid. Five-banded structure is seen  $\times 73,000$ . (Courtesy of C. E. Hall, M. Jakus and F. O. Schmitt and of *J. Appl. Physics* 16 263 1945)

scope. It is to be hoped that the association of electron microscopy and x ray diffraction, along with the application of biochemical methods, may permit a greater knowledge of the chemical nature of the periodic ultrastructure, not only in the collagenous fibers but also in other biological materials.

When collagenous fibers are treated with acid or with pepsin, they fragment longitudinally into fine filaments which retain their periodicity in the axial direction. These filaments, once dissolved in acetic acid, can be made to pass through filter paper and afterwards upon neutralization, under certain conditions, actually to reorganize themselves into new fibrillae with a periodic axial structure.

This observation is of considerable interest in relation to the problem of fibrogenesis, be it extracellular or inside the protoplasm. It favors the hypothesis that in the case of collagen fibers this process probably depends on the aggregation, laterally and longitudinally, of smaller asymmetrical units. However, recent studies have demonstrated the possibility that other kinds of fibers might be formed by the aggregation of globular molecules and that this mechanism can be reversed by changing the condi-

tions of the medium. The important observation has been made that a soluble globular protein like insulin, when submitted to special conditions of temperature and pH, can be converted into fibrillae of indefinite length and of about 140 Å in width. Apparently the corpuscular molecules, with low degree of asymmetry, have linked together end to end and also laterally to make a columnar aggregation. This fibrous protein, by changing the medium, may be reverted again into the globular form without appreciable loss in biological activity (Vaughn).

Actin, a protein extracted from muscle (see Chapter XII) also may be reversibly transformed from the globular to fibrous state. It has been studied under the electron microscope (Figs 137 and 138) (Jakus and Hall). These observations suggest that similar processes of fibrous formation might occur inside the protoplasm or in the surrounding tissue fluid under special conditions and possibly by the intervention of enzymatic systems. This hypothesis seems particularly appropriate to explain the formation and rapid disappearance of cellular fibrous structure like the aster and the mitotic spindle. However, the process might prove to be of great importance also in other morphogenetic phenomena.

### Ultrastructure of Some Cellular Structures

The different types of fibrillar organization described above may be found in numerous cellular structures, such as cilia, flagella, myonemes of the Protozoa, tails of spermatozoa, chromosomes, mitochondria, fibers of the asters and spindles, in all of which there appears birefringence with polarized light.

Polarization optics permit us to demonstrate the existence of a fibrillar structure in places where the microscope does not reveal any fibrillae *in vivo*. Such is the case in the *axis-cylinder* of nerve fibers (see Chapter XII) and in the achromatic spindle. In both, after fixation, it is possible to see fibrillae, but these are not apparent *in vivo*. On the other hand, with polarized light and with the electron microscope there can be seen evidence of delicate submicroscopic fibrillae definitely orientated, which, upon fixation, may be aggregated into coarser elements visible with the microscope. This fact has a considerable theoretical interest, for it demonstrates that many artifacts of fixation are only the exaggeration of an underlying submicroscopic organization, which is revealed by the coalescence of the micelles and by the loss of the water of imbibition produced by the fixatives.

The *vibratile cilia* and their *intracellular roots* show positive birefringence, indicating the existence of particles orientated in

## Microsomes

It is now over twenty years since the observation was made with the ultramicroscope that the hyaline matrix of some cells (such as amebae) contained small particles showing active brownian movement. The diameter of these particles is very much

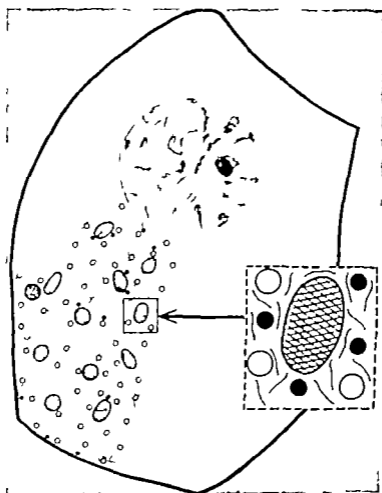


Fig. 31 Diagram of the organization of a hepatic cell showing the nucleus, the cytoplasm and the membrane. The chondriosomes (mitochondria) are represented by large bodies with cross-hatching; the glycogen microsomes, by hollow spheres; the lipoprotein microsomes, by solid spheres; and the micelles responsible for the sol-gel changes (structural proteins) by filamentous lines. (From Lazarow)

below the limit of resolution of the microscope. Recently thanks to the development of new methods, the interest of investigators has been concentrated anew on these submicroscopic particles.

If tissues are triturated very finely in such a way as to destroy the cellular membranes and are then suspended in physiological salt solution and centrifuged, one can isolate parts of the cell of varying size according to the force employed (*differential or fractional centrifugation* of Bensley). Thus it has been possible to

isolate nuclei, mitochondria and, recently submicroscopic particles, the *microsomes* of Claude (Fig 31) These are corpuscles of 500 to 3 000 Å, with a lipoprotein composition of complex nature in which are included phospholipids and ribonucleoprotein These microsomes have been separated in the ultracentrifugation of numerous tissues Microsomes contain 2 per cent mositol, 40 to 45 per cent lipids, two-thirds of which are phospholipids The high phosphorus content (1.5 per cent) reflects the abundance of phospholipids and ribosenucleotides They contain also sulfur iron and copper Of the various enzymes and enzyme systems studied, apparently no one has yet been found to be associated with the microsomes However cytochrome C was found in appreciable amounts (Claude) and also the thromboplastic activity present in microsomes (Chargaff) Recently a second type of submicroscopic particle has been isolated from hepatic cells This type seems to be composed essentially of glycogen with very little protein (Lazarow) These studies demonstrate that, besides the mitochondria and inclusions which are visible with the microscope, there exist in the cytoplasmic matrix a whole series of smaller particles, the *microsomes* of varying size, but of submicroscopic dimensions Another fact of great interest which these studies reveal is that the microsomes contain ribonucleoproteins (see Chapter VII) In this respect, these particles have some similarity to the viruses of plants, the virus of chicken sarcoma and of influenza, the bacteriophages, and so forth, which also contain nucleoproteins Claude believes that the microsomes may likewise have the power to divide and to take part in cellular synthesis

### Structural Proteins

Although the methods of polarization optics generally do not permit us to demonstrate the existence of a framework of fine submicroscopic fibrillae ("cytoskeleton" of Peters) which might support the protoplasm and permit an explanation of its mechanical properties, the existence of such fibrillae is suggested by many modern authors (Seifriz, Meyer) Such elongated micelles which are distributed through all the cytoplasmic matrix would be those responsible for the thixotropic changes (sol gel) which occur with frequency in the cells. If the protoplasm of a *Plasmodium* is drawn out with a microneedle, there will be seen to appear elastic and resistant fibers which offer resistance to the force exerted (Fig 32) During the formation of the pseudopodia birefringence may appear and in the fertilization of certain eggs bire-

fringence is seen at the end of three minutes after the entrance of the spermatozoon. All these facts and others which would be too many to enumerate, suggest the existence of elongated components, which would permit the maintenance of the structural relationships of protoplasm (Fig 31) At present it is considered that in the network formed by these structural proteins the polypeptide chains may be held together by cross linkages of hydrogen bonds or van der Waal forces, or even by stronger valences. *Changes in the strength of these cross linkages and in the degree*

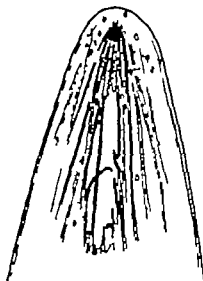


Fig. 32. Protoplasm of a Plasmodium drawn out with a microneedle (the large black point) which shows the appearance of fibrous structures in the ground cytoplasm. (From Seifriz.)

of folding or in the length or aggregation of the chains may produce the transformation of a sol into a gel and vice versa in a particular region of the protoplasm. These mucelles would not need to be constantly united to each other, for there might exist long range forces capable of holding them together although they would be separated by spaces of as much as 150 Å, thus maintaining the cohesion of the protoplasm \*

Bensley distinguishes another structural component, *ellipsin*, which is the material that persists after all the soluble proteins have been extracted and which would be the basis of permanent structures such as the cellular and nuclear membranes.

Recently experimental evidence has been presented of the possibility that long range forces between macromolecules may also be specific. Specific absorption of antigens and antibodies and the action of certain enzymes apparently take place even when the antigen or the substrate are separated from the antibody or the enzyme by blankets. These consist of up to ten monolayers of a dielectric substance (such as barium stearate, formvar) with a total thickness of 200 Å. (Rothen.)

In summary recent studies permit us to distinguish in the fundamental protoplasm a series of parts and of submicroscopic units, the interrelation of which results in the sum total of properties which we call vital (Bensley)

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## Chapter V

# MORPHOLOGY AND FUNCTIONAL SIGNIFICANCE OF THE CYTOPLASMIC ORGANOIDS

## CHONDRIOME

Under the generic denomination of *chondriome* are grouped all of the cytoplasmic bodies with the form of granules (mitochondria) or of rods or filaments (chondrioconts) which are found constantly in all plant and animal cells and which are characterized by a series of organoleptic, staining and physico-chemical properties. Among these the most important are their visibility in vitro, their supravital coloration with stains such as Janus green, the necessity of special techniques of fixation for their preservation, and their lipoprotein composition.

Although described by Altmann (1890) who gave the name "bioblasts" to the granules which appeared in cells with his method of fixation and coloration, the chondriosomes had been observed earlier by other authors, for example, Flemming and Kölliker. All these works suffered, nevertheless, the effect of the wave of skepticism which followed the investigation of the action of fixatives on colloidal models (page 52) and remained in obscurity until Benda (1897) demonstrated anew the structures described by Altmann and Flemming and called them mitochondria (signifying thread and granule). These observations received definite confirmation when Benda was able to demonstrate the mitochondria in living cells, and when Michaelis (1900) applied supravital coloration with Janus green to them.

The terminology applied to this organoid is extremely complicated (according to Cowdry it has been given more than fifty different names). In the Anglo-Saxon countries they are called mitochondria (Gr. *Mitos* filament *Kondria*, granule) both as the entire group of organoids and as each unit in particular. The nomenclature generally used in the Latin countries gives the name *chondriome* to the group of organoids, *chondriosomes* to each one and we apply the name *mitochondria* and *chondriocont* to the granular and filamentous forms respectively.

### Vital and Supravital Examination

Although the immediate examination offers certain difficulties due to the low refractive index of the chondriosomes, these can be observed in numerous living cells and, in particular in those cultivated in vitro. They also are visible with the darkfield (Fig. 12).

The vital examination is particularly important in the study of the variations of the chondriome with time and under the action of different stimuli. It is seen thus that the chondriosomes have two types of motion: one, of agitation, and the other, of displacement from one part to another of the cell. Furthermore, it can be observed that the filaments fragment into granules and that these also unite together. The chondriosomes respond to changes in the osmotic pressure of the medium increasing in size in hypotonic solutions and diminishing in the hypertonic (Lewis and Lewis). This fact can be explained by supposing the existence of a semipermeable membrane on the surface of the chondriosome and salts in its interior, or as due to changes in the hydration of its organic components.

### Supravital Coloration

The immediate examination is much facilitated by coloration with a dilute solution of *Janus green*, in which the chondriosomes take on a greenish blue color, which later is reduced to red, and finally decolorization occurs. It has also been possible to color the mitochondria, in tissue cultures, with methylene blue, but this coloration is inhibited by potassium cyanide and is decolorized by a bright illumination.

The coloration with these stains and with others such as cresyl violet and toluidine blue may be related to the processes of oxidation-reduction, since they are indicators of the oxidation-reduction potential of the cell (see Chapter III).

### Fixation and Staining

It has long been recognized that the chondriome is a very labile structure which disintegrates very readily by the action of fixatives. For this reason, all the methods of fixation for the chondriome are based on the stabilization of this structure by the prolonged action of oxidizing agents, such as osmic acid, chromic acid, and potassium bichromate. As a stain, one usually uses iron hematoxylin (Regaud) or acid fuchsin (Altmann). In this latter method, one carries out an overstaining with heat and then decolorizes with picric acid, aurantia, or the like. This staining characteristic recalls that which is presented by the acid-fast bacteria, such as the tubercle bacillus and that of leprosy, which also are stained in warm fuchsin and resist the action of acids. This property is attributed, in these cases, to the presence of an external layer of lipoid or waxy nature and in the chondriosomes it was claimed that it could be due to a similar composition.

**Morphology (Form, Size, Distribution Orientation Number)**

The *form* of the chondriosomes, as was mentioned above, is variable, but in general is *filamentous* or *granular* (Fig 33) In some cells, and especially during certain functional states, there may be seen other forms derived from those mentioned. Thus, a chondriocont swells out at one end to assume the form of a *club* or hollows out at one end to take the form of a *tennis racket* (Fig 40) The mitochondria, at other times, may become *vesicular* by the appearance of a central clear zone. In the liver there have



Fig. 33 Chondriome in the hepatic cells of *Amblystoma*. Chondriosomes of variable form are seen. Fixation, Regaud. Stain, iron hematoxylin.

been described cycles in the morphological variation of the chondriome (Noël) In the majority of cells, the chondriosomes are filamentous, but in some, such as the spermatozoa, oocytes and mature eggs, they are granular In the cells of intestinal epithelium, they have a filamentous form in the apical part and at the sides of the nucleus, but a granular form in the basal cytoplasm.

The *size* of the chondriosomes also is variable but the fact is interesting that, in general, the diameter remains constant and that only the length varies This seems to indicate that growth occurs by the addition of material at the extremities

The *distribution* of the chondriome is in general uniform, but there are many exceptions to this rule. Thus, in the cells of the kidney they are aggregated, especially in the basal region next to the blood capillaries, and the same is true, although not to such a degree, in different glands of fixed polarity In some cases, they are accumulated preferentially about the nucleus or in the peripheral cytoplasm Such margination as well as the perinuclear

accumulation may be found in normal cells, but it is more frequent in pathological conditions. Some of these changes depend also on the overloading with reserve substances which displace the chondriosomes.

Frequently they are aggregated in a radial form about the centrosomes and among the rays of the aster. During cellular division, the chondriosomes tend to aggregate about the spindle, and upon the division of the cell are distributed in approximately equal quantity between the daughter cells.

The chondriosomes in some cells present a more or less definite *orientation*. Thus, in the cylindrical epithelial cells they are generally orientated in the basoapical direction, parallel to the main axis. In the hepatic cells of fish and amphibia they are orientated along the axes which unite the blood capillaries with the bile capillaries. On the other hand, in the leucocytes they are more or less radially arranged with respect to the center of the cell. Recently it has been suggested that these orientations depend upon the direction of the currents of diffusion within the cells and would be intimately linked to the submicroscopic structure (structural proteins, Chapter IV) of the fundamental cytoplasm (Pollister).

The *quantity* of the chondriosomes is difficult to determine but, in general, it can be said that this varies with the type of cell and the functional state of the cell (see below).

In summary, the morphology of the chondriome varies from one cell to another, but is more or less constant in cells which have a similar function. This indicates that the morphology of the chondriome is a fundamental property connected with the organization of the cell (Cowdry).

### Structure and Chemical Composition of the Chondriome

From the physicochemical point of view, the chondriome may be considered as a more gelified part of the cytoplasm. With micromanipulation it has been possible to demonstrate that the chondriosomes are relatively stable and are displaced by the microneedle without suffering changes.

The *specific gravity* is greater than that of the fundamental cytoplasm. This property can be studied using the method of *ultracentrifugation* which consists in submitting the cells to a very great centrifugal force (200 000 to 400,000 times the force of gravity) using a rotor driven by compressed air. The different structures of the cytoplasm and of the nucleus are stratified and those in which the specific weight is greater (as happens in the case of the chondriome) are deposited in the centrifugal pole.

Even after being displaced, the chondriosomes maintain intact their form and structure, a fact which demonstrates also that they are stable and relatively rigid elements

The knowledge of the *chemical composition* of the chondriome has advanced considerably in the last ten years, thanks to the employment of new techniques. Before that time it was considered, without a very secure basis, that the chondriosomes were composed usually of phospholipids. This idea was based on the solubility (disappearance) of the chondriosomes with the different fat solvents, the stabilization by means of chromatization, the staining properties, the impregnation with osmic acid which can be observed in certain cases, and the parallelism which exists in a particular organ between the quantity of phospholipids and of chondriosomes

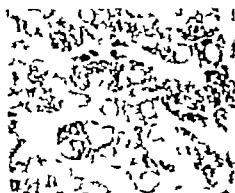


Fig. 34

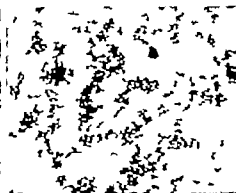


Fig. 35

Fig. 34. Chondriome in hepatic cells of a mammal. Fixation, Regaud. Stain, from hematoxylin.

Fig. 35. Chondriome of hepatic cells isolated by fractional centrifugation. (From Lazarow)

In hepatic cells, frozen and dried by the method of Altmann Gersh (Chapter III) it was possible to study in ideal conditions, the solubility with different solvents and the chemical properties of the chondriosomes. If the tissue is extracted for sixty hours with fat solvents in the Soxhlet apparatus the chondriosomes are stained in the same manner as in normal material. This seems to refute the theory that the staining properties of the chondriome are due to its lipid content. Furthermore, in these conditions, the Millon reaction for proteins and the digestion with pepsin give positive results for the chondriosomes, a fact which suggests that proteins constitute the most important component (Bensley and Gersh)

Later thanks to the discovery that the chondriosomes of the hepatic cells of certain species were insoluble in saline solutions,

it was possible to isolate them by *differential centrifugation*. At first, the nuclei and other parts of the cell are separated and then the chondriosomes precipitate out and can be "purified" by repeated washings and centrifugations (Figs 34 and 35) (Bensley and Hoerr)

The chemical analysis of the chondriosomic fraction revealed the following composition (Bensley)

Proteins and unrecognized substances	65%
Glycerids	29%
Lecithin and cephalin	4%
Cholesterol	2%

These results show that, at least in the hepatic cells, the chondriome has a lipoprotein composition in which the protein part predominates. In the lipoid fraction the glycerids are found in the greater proportion. Recently, in a detailed chemical study of the mitochondria of the liver, the conclusion has been reached that these are composed principally of proteins, the lipids representing only 25 per cent (Claude). Nevertheless, in this latter fraction, from 75 to 80 per cent is phospholipid.

In spite of the high lipoid content, the chondriosomes in general are not impregnated with osmic acid, nor do they stain with Sudan III, by which fact one must suppose that the fat is found in a very fine ultramicroscopic state of dispersion. As a consequence of these studies, the conclusion was reached that the chondriosomes are coacervates, that is to say structures which result from the aggregation of molecules, the coherence of which is maintained by means of intermolecular forces of attraction, and that the superficial part of the chondriosomes is constituted by a mosaic of protein and lipoid micelles. Any agent capable of changing the state of equilibrium of the forces which maintain the cohesion of such a structure can provoke the dispersion of its molecular units. This would explain the lability of the chondriosomes under the action of certain agents and their disappearance with many fixatives and organic solvents.

Besides the components cited above, it has been possible to demonstrate the presence of an appreciable quantity of ribonucleotides. The content of sulfur is relatively high (0.82 to 1.16 per cent) and it probably occurs in the form of SH groups, since the mitochondria give a positive reaction with nitroprusside. These SH-groups may belong to the proteins and also be due to the presence of glutathione (Bourne, Joyet Lavergne). The content of iron, which is due to the presence of cytochrome compounds, is approximately 0.2 per cent and that of copper from

0.02 to 0.035 per cent. By histochemical methods and also by means of the technique of separation of the mitochondria, it has been possible to demonstrate the presence of various enzymes and vitamins. The possibility of the existence of proteolytic enzymes in the mitochondria has been suspected due to their specific staining with Janus green B which precipitates in the presence of such enzymes. Recently it has been demonstrated that the chondriome contains or is associated with the greater part of the cytochrome oxidase and succinic dehydrogenase of the cytoplasm (Hogeboom, Claude and Hotchkiss see Chapter X).

The presence of vitamin A in the mitochondria was suspected from an early period due to the yellowish coloration which they have in certain lower animals (Gatenby). Furthermore, it has been observed that they give a positive reaction with antimony trichloride which is characteristic of the carotenoids in general (Joyet Lavergne). In isolated mitochondria a content of 249 to 910 U.S.P. units of vitamin A per 100 mg. of lipid material has been found (Goerner). With the histochemical technique of acetic silver nitrate it has been possible to show in some cases the presence of vitamin C in the chondriome (Bourne, Leblond). Inositol also has been found in it.

The topographic distribution of all of these components would be, according to Bourne, as follows:

1. A superficial layer composed of a lipoprotein mosaic.
2. A cortical region poor in water where the proteins, fats, lecithin, cephalin, cholesterol, and other fat soluble substances, such as vitamin A, predominate.
3. An internal region rich in water where there would be accumulated proteins, vitamin C, glutathione, oxidases, and so on.

Nevertheless, this conception is, at present, highly theoretical and needs to be confirmed by means of further histochemical and submicroscopic studies.

### Submicroscopic Structure

The ultrastructure of the mitochondria has been studied but little up to the present. In the intestinal cells of *Ascaris* it has been found with the polarizing microscope that they show a uniaxial positive birefringence. Also in the rodlets of the distal convoluted tubule a positive birefringence has been found which could be inverted by immersion in a medium with a greater refractive index (Grave). This suggests the existence of protein chains orientated in a direction parallel to the direction of the mitochondria and of lipids orientated perpendicularly. This has

been confirmed in living cells after vital staining with chrysoidin (Monné) The study of the mitochondria with the electron microscope has encountered the difficulty of the considerable thickness of these structures and of their high electronic density In cells

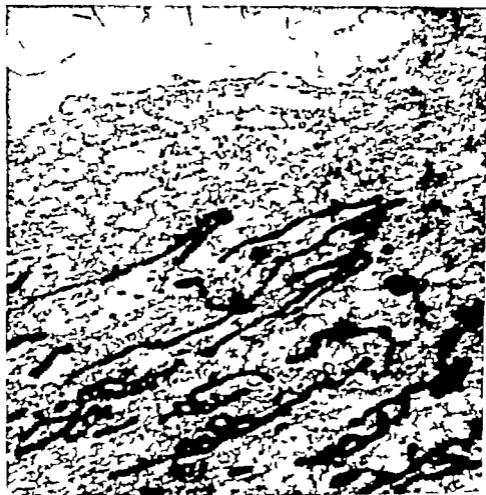


Fig. 36. Electron micrograph of the margin of a cultured rat fibrocyte. Chondriosomes show internal bodies and a surrounding sheath. Vesicular material is seen in the endoplasm. The outer layer is free of granules Osmic acid fixation.  $\times 7000$  (Courtesy of K. R. Porter)

cultivated *in vitro* and fixed in osmic acid, the mitochondria are distinguished from the cytoplasmic matrix by their greater electron density (Fig 36) (Porter Claude and Fullam) In some cases, one sees masses of greater density distributed along the length of the mitochondria and having a diameter of 10 to 20  $\mu$  (Fig 37) The sickle-shaped mitochondria of the spermatozoa can be separated and in this condition also they show an internal structure (Schmitt)

The mitochondria isolated by means of differential centrifugation appear in the electron microscope as opaque bodies which

scatter the electronic beam uniformly. In some cases, one may observe small elements within the mitochondria of greater density and having a diameter of some 80 to 100 m $\mu$  (Claude and Fullam)

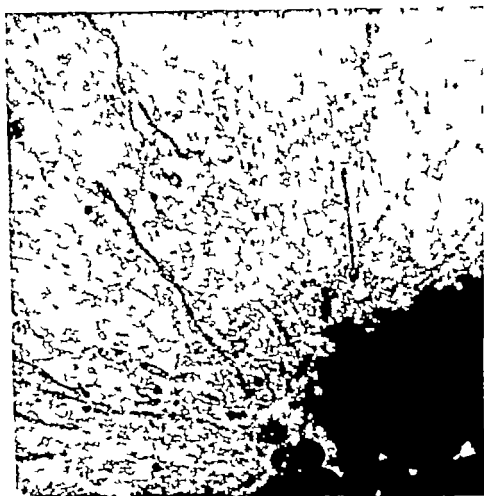


Fig. 37 Electron micrograph of a portion of a cultured chick macrophage. In the lower right corner of the globules "stained" with osmium can be seen. Radiating from this point are chondriosomes, which show internal structure and endoplasmic strands.  $\times 5200$ . (Courtesy of K. R. Porter)

### Functional Significance of the Chondriome

Although all cytologists are in accord in considering the chondriome as an essential part of the cytoplasm with an important role in cellular physiology, the exact nature of its function is little known. We can affirm that in this case, as in many other aspects of cellular life, the possibilities of the morphological method have been practically exhausted and that one must hope for the development of new cytochemical methods to solve entirely the problem of the functional significance of the chon

driome In the enormous literature there exists, nevertheless, a large array of data which permits us to suspect the intervention of the chondriosomes in the metabolic processes of the cell, although the intimate nature of this intervention escapes the present methods of cytological analysis A great number of these evidences are *indirect* and are based upon the variations which are produced in the morphology of the chondriome in the different phases of cellular life

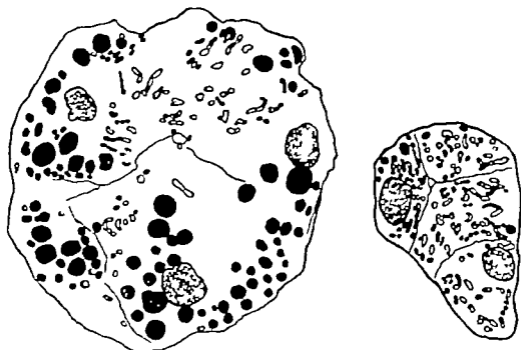


Fig. 38. *Right* Hepatic cells of the normal toad (*Bufo arenarum* Hensel) Fat drops in black, chondriosomes clear with a dark border nuclei stippled, chondriosomes with a lipoid deposit are seen at the base of the cells. *Left* Hepatic cells of a hypophysectomized toad. Diminution of the fat drops and increase of the chondriosomes. Fixation, Champy Stain, Altmann's fuchsin.

Thus, for example, in glands in *active secretion*, there is, in general, a hypertrophy of the chondriomes characterized by an increase in the number and size of the chondriosomes. On the other hand, in those processes in which there occurs an increase in the quantity of fat or glycogen, there is a diminution of the chondriosomes, parallel to this increase (Fig 38) In general, where there are many drops of fat, there are few chondriosomes, and vice versa These facts have been interpreted as evidence of a *relation between the chondriosome and the accumulation of fats or of glycogen*

A similar variation which proceeds up to the disappearance of the chondriosomes is seen in the *formation of the plastids* of plant cells In this case, the phases which lead to the trans-

formation of one organoid into the other appear to be well established. A similar fact is observed in the erythroblasts in which, as the quantity of *hemoglobin* increases, the chondriome diminishes. In the mature erythrocytes the chondriome may disappear completely (Fig 39)



Fig. 39 Evolution of the chondriome in the erythrocytes of an amphibian. From left to right, the chondriome diminishes, while the content of hemoglobin increases. Fixation, Regaud. Stain, iron hematoxylin. (From Rojas and De Robertis.)

The chondriome of the hepatic cells suffers wide variations according to the state of nutrition of the animal and the type of food administered (Noël). In the fishes, these changes are very conspicuous and are seen a few hours after ingestion. The filamentous chondriosomes are modified, and club-shaped and racket shaped forms appear as well as vesicular mitochondria at the same time, the cytoplasm becomes vacuolated. At forty-eight hours, these processes have ceased and the former aspect is recovered (Fig 40)

Another group of data comes from the observation of a

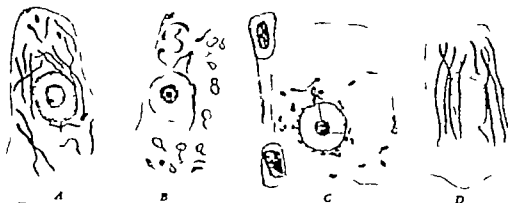


Fig 40 Chondriome of the hepatic cells of *Crasterodon decemmaculatus* in different stages of nutrition. A, at the time of ingestion of the food, chondriosomes B at nine hours, chondriosomes shorter with club shaped and racket type forms; C, at twenty four hours, increase and clearing of the cytoplasm with short chondriosomes around the nucleus D at forty-eight hours, similar to A long chondriosomes. Fixation, Regaud. Stain, iron hematoxylin (From Rojas, De Robertis and Castellengo)

topographical relationship between the chondriosomes and the different products absorbed or elaborated by the cell. Various authors, since the time of Altmann, have observed that the *deposit of lipids* may occur in the chondriosomes (Fig. 38). In the hepatic cells of the amphibia this process is seen especially in the small mitochondria situated near to the vascular pole on which the fat forms a type of crescent or peripheral cap which is impregnated by osmic acid or stained with Sudan III. Once formed, the drops of fat increase progressively in size toward the opposite pole.

In other cases, the accumulation of different substances such as fat, hemoglobin and protein occurs in the interior of the chondriosomes. In the hepatic cells of the amphibia there may be observed in certain states the accumulation of a pigment derived from the hemoglobin within vesicular chondriosomes. In these cases, the reaction for ionic iron was positive in some chondriosomes (De Robertis).

According to Cowdry, in all these processes of accumulation of substances, the chondriome would intervene especially as an *interfacial film*. On its surface there would be adsorbed and concentrated those substances which later could pass to the interior of the chondriosome, where they would be deposited as such or would undergo chemical changes by enzymatic action.

We have seen, above, that the chondriome shows a great lability and is readily altered by the action of various agents. This fact is seen particularly in pathological processes in which it can be affirmed that the chondriome is one of the most sensitive *indicators* of any sort of injury brought about in the cell. Most frequently one sees the fragmentation of the chondrioconts and the disintegration of this organoid, but in other cases, as in scurvy, the mitochondria fuse together to form voluminous bodies called *chondriospheres* (Bourne). For a long time, although with little basis, the idea has been advanced of the intervention of the chondriome in *cellular oxidations*. Recently, thanks to the isolation of the chondriome by fractional centrifugation, it has been possible to test this problem directly and it has been found that the chondriome actively oxidizes glutamic acid, likewise oxidizes succinic acid, although with less intensity, and gives, with Nadi's reagent, a positive reaction for cytochrome oxidase (Lazarow and Barron). Although these results are as yet preliminary they demonstrate that the chondriosomes contain some of the enzymes which take part in cellular oxidations. We have mentioned above recent results which show that the chondriome contains a large amount of cytochrome oxidase and of succinic dehydrogenase.

Furthermore, the consumption of oxygen of a piece of liver which has been frozen is below that of a piece of normal liver a fact which coincides with the disintegration of the chondriome which takes place in the former. This fact perhaps could mean that in the chondriome there exists a concentration of respiratory enzymes which facilitates the transport of hydrogen (and therefore the consumption of oxygen see Chapter X) and that when the chondriome disintegrates, such concentration is lost with the resultant fall in the oxidations (De Robertis and Novinski)

In the Protozoa, observations have been made which permit us to suspect an enzymatic action of hydrolytic type. In the amebae the food incorporated is brought into intimate contact with some chondriosomes. Later a vacuole is formed which encloses the particle of food and the adjacent chondriosomes. If the process is followed, it is seen that, with the progress of digestion of the food, the chondriosomes diminish progressively in size and at the end of three hours disappear completely (Hornig). In the germination of cereals it has been seen that the secretion of diastase seems to be related likewise to the chondriome.

In *summary* although the function of the chondriome remains almost unknown, this organoid does seem to take a certain part in the different processes of cellular metabolism. In these processes, the chondriosomes behave like interfacial membranes where there would be concentrated various substances and, furthermore, as carriers of hydrolytic and oxidizing enzymes. The study of the functional significance of the chondriome awaits the development of new cytochemical methods and the more intensive employment of those already in existence, such as fractional centrifugation, followed by chemical and enzymatic analysis.

## THE GOLGI APPARATUS

The name *Golgi apparatus* or *internal reticular apparatus* is used to designate certain reticular granular or irregular structures, almost always localized or polarized, which are fixed preferentially with fixatives which stabilize the lipoids and which are impregnated by silver salts or by prolonged treatment with osmium tetroxide.

In spite of the fact that in the last fifteen years considerable advances have been made in the study of the composition and function of the Golgi apparatus, it is not yet possible to give a definitive interpretation of its nature and functional significance.

This organoid was discovered by means of the silver method by Golgi (1898) in nerve cells of the barn owl and of the cat

and later found with the same reticular aspect in almost all of the differentiated cells of vertebrates.

In the invertebrates and in certain stages of development of vertebrates, there appear individual corpuscles which are impregnated in the same manner as the Golgi apparatus and which have been homologized with this organoid. These Golgi bodies or golgiosomes (Sosa) have received numerous denominations

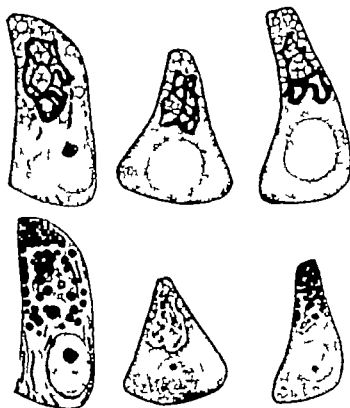


Fig. 41 Above Three acinous cells from the pancreas of the guinea pig impregnated by osmic acid to demonstrate the Golgi apparatus. Below The same cells, after decolorizing and restaining with iron hematoxylin, show clear canals which correspond to the Golgi networks. In the two cells on the left, chondriosomes also are seen. (From Cowdry)

(lepidosomes, lipochondria, dictyosomes, and so on) which have made their homologization very confused. Nevertheless, it is evident that there is an intimate relationship between the two types of structure.\*

The concept of the Golgi apparatus was complicated also by the discovery more or less simultaneous (Holmgren, 1899) of clear canals situated in the cytoplasm of many cells (trophospongium) the aspect and position of which was believed similar

The nomenclature of the Golgi apparatus is extremely complex. That proposed by Sosa (1930) presents advantages by its simplicity and dynamic character

*Golgiokinesis* Division of the Golgi apparatus during karyokinesis. (Synonym dictyokinesis) (Perroncito)

*Golgiosome* Corpuscles resulting from golgiokinesis. Formations described in the

to that of the Golgi apparatus. So great was this homologization that Cajal called this organoid the "nets of Golgi Holmgren."

At the present time, the most frequently accepted opinion is that there is no connection between the two structures. In many cases it has been possible to demonstrate them simultaneously in the same cell and they have even been separated with ultracentrifugation by means of their different specific gravities. It cannot be denied, nevertheless, that in many cases the clear canals that are observed in stained cells are the *negative image* of the Golgi apparatus (Fig. 41)

Later a greater complication arose with the theory of the vacuome, developed principally by Parat and Paillevé. This theory supports the view that the two essential components of cytoplasm, in plant as well as in animal cells, are the chondriome and the vacuome. This latter structure is constituted by vacuoles which stain with neutral red in vital coloration. According to these authors, the Golgi apparatus would be nothing more than an artifact which results from the precipitation of silver or osmium salts between the vacuoles or on the surface of the same.

The theory of the vacuome has been practically discarded in recent years. There are numerous cytological observations which have demonstrated in a conclusive form that the two structures are independent, although sometimes there may exist between them a mere spatial relationship. It is admitted at the present time that neutral red, in staining the cell, accumulates first on granules and then, secondarily gives origin to the vacuoles (*crinome* of Chlopin). This fact, easily visible in supravital observation, demonstrates that the vacuome is a secondary structure called forth by the coloring agent. According to some authors, the granules which are colored at the beginning by neutral red would represent the presubstance of Golgi and not the true Golgi apparatus.

### Vital or Supravital Examination

In the great majority of cells it is not possible to distinguish in the living state any structure similar to the Golgi apparatus. There are, however, some exceptions to this rule, for different authors have been able to see it in the cells of the prostate, in intestinal cells of *Ascaris*, in Purkinje cells and in various tissues of the invertebrates. Furthermore, it has been demonstrated in cultures stained with methylene blue and it has been vitally stained with trypan blue. Further it has been observed recently and photomicrographs have been made of it, in the cells of the seminal vesicle and in spermatids, by means of the phase contrast microscopy (Brice, Jones, and Smyth). With vital or supravital staining by methylene blue it has been shown that the Golgi apparatus can be seen in vivo in numerous types of cells in both the invertebrates and vertebrates (Worley).

The fact that generally it is not visible in vivo caused many authors to doubt the real existence of this organoid. The argument

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invertebrates as the Golgi apparatus. (Synonym dictyosomes) (Perroncito)  
*Golgiolysis*: Process of dissolution of the Golgi apparatus.  
*Golgiorrhexis*: Fragmentation of the Golgi apparatus.  
*Golgiogenesis*: Formation and ontogenetic differentiation of the Golgi apparatus.  
*Golgiocytoarchitecture*: Study of the cytoarchitecture in relation to the Golgi apparatus. (Sosa, J. M. Aparato de Golgi y nomenclatura celular—Act. del Cong. Int. de Biología de Montevideo, 1930 p. 1955)

of its invisibility is not, however valid, since this may signify only that the Golgi apparatus has the same refractive index as the rest of the cytoplasm. When one has a certain experience in the study of this organoid and particularly when one considers it cytophysiologically, that is, considered as a dynamic structure which varies with different functional states of the cells, then all doubts concerning its real existence vanish. We may affirm with Kirkman and Severinghaus that the most conclusive proof that the Golgi apparatus is an organoid is not the universality of its presence in fixed preparations, nor the observation of it in living material, but rather, its behavior when one observes it in different physiological conditions. This behavior is so characteristic and constant that one can have no doubt with regard to the reality of the substance or structure which shows it.

### Fixation and Staining

In the definition it was said that one of the essential characteristics of the Golgi apparatus is its impregnation by silver salts or with osmium tetroxide. The first requires a previous fixation by formol, to which is added uranium nitrate or cobalt nitrate. Both techniques are relatively complicated and in order to obtain constant results it is necessary to adapt them to each type of tissue.

### Morphology (Form, Size Position)

The *form* of the Golgi apparatus varies considerably from one cell to another and even in the same cell, with the functional state this extreme variability makes one think that in the living cell it may be undergoing a slow but constant change. It is described, in some cases, as a dense reticulum of anastomosing trabeculae, an irregular fenestrated plaque, a ring hollow spheres united together and so on. In the nerve cells it is arranged, in general, in the form of a reticulum of wide meshes around the nucleus and occupies an extensive zone of the cytoplasm (Fig 42).

In the majority of the exocrine glands it forms a dense reticulum of coarse meshes, situated between the nucleus and the excretory pole and more or less intermingled with the product of secretion (Fig 42). In the leucocytes it is a dense mass located in the concavity of the nucleus.

The *form* of the Golgi apparatus is, in spite of its variability characteristic for each type of cell—a fact which demonstrates that it is a morphological character intimately linked to the function and organization of the cell.

In many cases, the Golgi apparatus may lose its reticular form and fragment into more or less coarse granules which are dispersed in the cytoplasm. This happens in certain functional states, but with greater frequency in pathological conditions. The fragmentation into fine granules (golgiosomes) occurs normally during mitotic division, a process which leads to a dispersion of

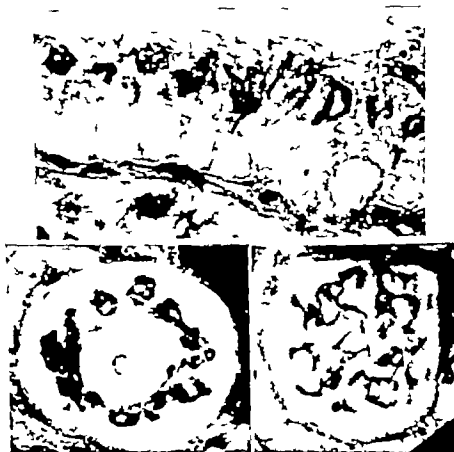


Fig. 4 Above Golgi apparatus in cells of the thyroid gland of the guinea pig, apical position. Osmic impregnation. Below left Ganglion cell, perinuclear Golgi apparatus. Below right The same, optical section tangential with respect to the nucleus. Silver impregnation.

the material which the Golgi apparatus contains and to an approximately equal redistribution between the daughter cells.

The size is likewise very variable. It is great in the nerve and gland cells and small in the muscle cells. In spite of the fact that it is in practice very difficult to appreciate quantitatively the variations in size of the Golgi apparatus, there remains no doubt that these exist and frequently are linked with the functional state, for example hypertrophy in hyperfunction and atrophy in hypofunction. In general, the Golgi apparatus is well

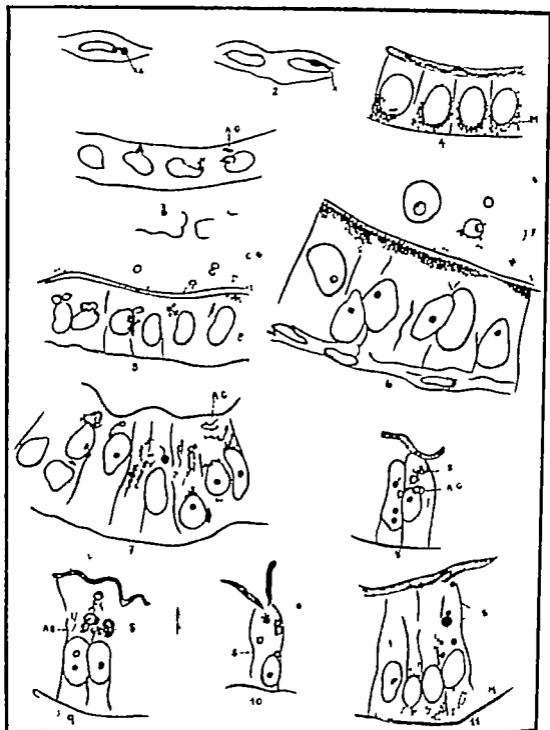


Fig. 43 Evolution of the Golgi apparatus (AG) in the follicular cells of the ovary of a teleostean fish 1 and 2, endothelial cells, AG at one end; 3 cuboidal cells, AG in apical position, 5 and 6 fragmentation of AG and penetration of the golgiosomes into the ovocyte; 7 reconstitution of AG 8 9 and 10 follicular secretions in relation with AG 4 and 11 chondriosomes. (From Rojas and De Robertis)

developed while the cell is in an active state, when it grows old, it progressively diminishes in size and disappears.

The position of the Golgi apparatus is relatively fixed for each cell type. In the cells of ectodermic origin the Golgi apparatus is, from the time of the embryonic state, polarized between the nucleus and the periphery (Cajal).

In the secretory exocrine cells which have, in general, a typical polarization, the Golgi apparatus is found between the nucleus and the excretory pole (Fig. 41). In the endocrine glands the polarity of this organoid is variable, except in the thyroid, where it is orientated toward the center of the follicle (Fig. 42). Due to some observations in which the Golgi apparatus of the thyroid cells appeared inverted in regard to its ordinary position, much importance was given to this organoid as an index of *secretory polarity* (Cowdry). This concept has received little confirmation and although in the case of the thyroid there can be recognized, by more direct means, the existence of an inversion of functional polarity (Gersh and Caspersson, De Robertis) this does not correspond, in the majority of cases, to a change in the position of the Golgi apparatus. On the other hand, the displacement of this organoid in cells may respond purely to mechanical factors.

In Figure 43 an interesting example is shown to illustrate the morphological variations which the Golgi apparatus may undergo in a single cell type in different stages of its evolution. The figure shows follicular cells, epithelial elements which surround an oocyte in a teleostean fish. These cells, as the ovum grows, are modified and pass from an endotheliiform state (Fig. 43, 1 and 2) to become a high cylindrical epithelium (Fig. 43, 4 to 11). At the same time, the Golgi apparatus undergoes very characteristic modifications. In the endotheliiform cells, it is small and is found at one of the poles of the nucleus (1) later before the morphological axis of the cell has changed, it abandons its polar position and is located between the nucleus and the ovum (2). In cells of greater height (cuboidal) the Golgi apparatus becomes more complex (3) and then commences to fragment into fine osmophilic granules (golgiosomes) (5). In more advanced states, the fragmentation is total (6). Simultaneously the golgiosomes infiltrate into the interior of the oocyte crossing the vitelline membrane (5 and 6). In higher cells the Golgi apparatus reappears as such (7) and commences in them a new functional state characterized by an intense secretion of products elaborated in intimate relationship with the Golgi apparatus (8 to 10) and which are excreted in the oocyte (follicular yolk).

### Structure and Physicochemical Properties and Chemical Composition

The physicochemical properties and the chemical composition of the Golgi apparatus could be studied up to the present only in an indirect way. A great advance in the study of the former came with the employment of the technique of *ultracentrifugation*. By submitting cells of the uterine glands to a centrifugal force 100 000 times gravity, one can displace the Golgi apparatus from

its normal position, toward the centripetal pole (Beams and King) This experiment demonstrates that the Golgi apparatus is a well defined cytoplasmic material and that it has a *specific gravity* less than that of the fundamental cytoplasm

Although the mere observation of impregnated preparations (Fig 42) could, by the clearness of its outlines, suggest the idea of a relatively rigid structure, the *consistency* of the Golgi apparatus seems to be relatively fluid. Indeed, if certain cells (intestinal) are crushed under the microscope, the mitochondria and granules are freely displaced toward the zone occupied by the Golgi apparatus Further introducing *microneedles* into the cytoplasm, one encounters no resistance in this region, a fact which might indicate the absence of a firm network

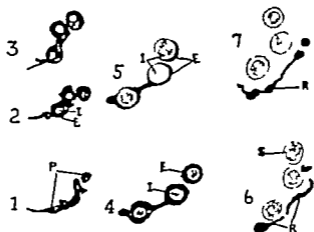


Fig. 44 Diagram by Hirsch of the relation between the Golgi apparatus and secretion. E, externum, I internum; P presubstance R, residue of the Golgi apparatus, S secretory product. (From Hirsch)

The consistency of the Golgi apparatus appears to be, nevertheless, variable, according to the cells and perhaps to the functional state The results of ultracentrifugation are in favor of this conclusion. Thus, while in the uterine glands the consistency seems to be very fluid because the Golgi apparatus not only is displaced, but also is deformed, in the thyroid cells (Hellbaum) and in the cells of the spinal ganglia (Brown) it maintains its form although it changes in position, a fact which indicates a greater rigidity When one transplants centrifuged adrenal cells, the Golgi apparatus soon recovers its normal position (Dornfeld)

Various authors have considered the Golgi apparatus as composed of two parts, an external argentophilic and osmiophilic part and an internal argentophobic and osmiophobic part (literature in Bourne) This internal zone ('internum' of Hirsch) would be richer in water and less dense than the external part

("externum") According to Hirsch, besides the Golgi apparatus composed of these two parts, there exist homogeneous granules which he considers as precursors of the Golgi apparatus, constituting the presubstance of Golgi (theory of the presubstance system of Golgi, of Hirsch) (Fig 44)

According to Hirsch, each particle of presubstance is transformed into a true Golgi body when one can distinguish its two constituent parts, externum and internum. The presubstance is stained by methyl red and also with Janus green B, this having led to the supposition of a certain relationship between it and the mitochondria. The chromophobic part of the Golgi apparatus may be separated from the chromophilic part by means of centrifugation since they take up different positions in accordance with their different specific gravities (Richter)

The study of the submicroscopic structure of the Golgi apparatus has barely started. By means of polarized light, it has been possible to observe *in vivo* the Golgi apparatus of spermatocytes of certain molluscs. It has a lenticular form and shows in its externum a positive birefringence in the direction of its thickness, which indicates a radial orientation of lipids. This birefringence increases when it is stained with vital stains such as rhodamine B and chrysoidin. In this case, the internum did not show signs of a preferential orientation (Monné). In cells cultivated *in vitro*, fixed with osmic acid, and examined with the electron microscope, the Golgi bodies appear as dense structures characterized by rather angular outlines. Their high density after treatment with osmic acid has not permitted the distinguishing of any internal structure, if such exists (Porter, Claude, and Fullam). Nevertheless, in mononuclear leucocytes, without staining with osmic acid, likewise examined under the electron microscope, it has been possible to see that the zone occupied by the Golgi apparatus appears clearer than the rest of the cytoplasm and has a canalicular aspect (Fig 45) (Sosa). This lesser density to the electron beam would perhaps indicate a lesser density or molecular concentration. This apparently is in accord with the results obtained with the ultraviolet microscope (Hibbard and Lavin). Indeed, in photomicrographs taken with 2537 Å, the density of the zone of Golgi appears less than that of the rest of the cytoplasm. The apparently discordant results mentioned in the first place could easily be explained by the considerable affinity which the Golgi apparatus has for osmium tetroxide.

Very little is known of the *chemical composition*. Unfortunately we have not been able to isolate this organoid as we can the chondriome, and for this reason, any quantitative information

respecting its composition is lacking. For a long time, the idea has been advanced that the Golgi apparatus is composed of lipids. To arrive at this conclusion, various authors considered principally the capacity which it has to be impregnated with osmium tetroxide. This is, however, disputable since osmium tetroxide may be reduced by other substances and besides, the impregnation of fats is, in general, much more rapid than that of the Golgi apparatus. Another argument, somewhat more important, is the fact that in a cell treated with fat solvents, one cannot demonstrate the Golgi apparatus. Furthermore, its smaller specific gravity could perhaps be attributed to a lipid nature.



Fig. 45 Electron micrograph of the Golgi zone of the mononuclear leucocyte. The opaque zone below and to the left corresponds to the nucleus. In the cytoplasm there appears a clearer zone of canalicular nature with the same localization and aspect as the Golgi apparatus.  $\times 10,000$ . (Courtesy of J. M. Sosa.)

The application of histochemical methods for the demonstration of fats (Sudan III, scarlet red, Nile blue sulfate, and the like) gives, in general, a negative result. This, of course, does not signify that lipids do not exist. The negative reaction could be due, as in the case of the chondriome to the intimate association of the lipids with proteins (masked lipids, Chapter II). For this reason, the idea has been sustained that proteins also take part in the composition of the Golgi apparatus.

Dissociation of the protein and the lipids can be caused by using proteolytic enzymes which digest the protein and leave the lipid fraction free (Ciaccio). With this method it was found that the Golgi apparatus does stain with Nile blue sulfate, which is a stain for fats and lipoids. This interesting property seems to indicate that this organoid is of a lipoprotein constitution and that it has a high lipid content (Tarao). The presence of lecithin and cephalin in the Golgi apparatus of spermatocytes also has been claimed (Baker). According to some authors, the protein

part would be concentrated particularly in the internal zone of the Golgi apparatus

Recent studies seem to demonstrate that a relationship exists between the Golgi apparatus and vitamin C. This conception is based on a numerous series of studies on different cells in which the localization of vitamin C, demonstrated histochemically by the silver nitrate in acetic solution, is compared with the position of the Golgi apparatus, and which demonstrate that there exists a topographical coincidence between the two elements (Girood, Leblond, Bourne, Tomutti, and others). In the excellent monograph of Bourne, one may find a detailed bibliographical analysis on this subject.

In order for this conception to have value, it must rest on the foundation that the reaction used for vitamin C is completely specific and that the method cannot impregnate other substances or structures. Recently the value of this histochemical method has been placed in doubt both in connection with its specificity (Sosa) and its use in determining the intracellular distribution of vitamin C. Experiments with models (emulsions) would appear to indicate that the acetic silver nitrate tends to precipitate at the interfaces and this would explain the tendency of the Golgi apparatus to be impregnated by this method (Barnett and Fisher).

### Functional Significance of the Golgi Apparatus

As in the case of the chondriome, the problem of the functional significance of this organoid remains yet to be solved. The little that is known up to the present is based on indirect observations and more or less hypothetical deductions.

One of the facts which seems to be well established is that in many secretory cells there exists a relationship between the Golgi apparatus and the secretion, but the intimate nature of this relationship is practically unknown. This relationship was discovered by Cajal (1914) in the goblet cells, salivary glands and in the exocrine pancreas activated by secretin. Later, other investigators confirmed these observations, determined better the intermediate steps and made this a general concept for many other glandular cells.

Figure 43 (8 9 10) shows in the follicular cells of the ovocytes a typical example of this relationship. It is seen that in intimate association with the Golgi apparatus there appears a fuchsinophilic secretion substance in the form of rounded corpuscles (8 9). The secretion granules are surrounded by an osmiophilic ring more or less complete (8) or by a reticulum with nodal points (9). Finally the Golgi apparatus completely fragments and it may be seen that the secretion granules have osmiophilic parts adhering to their surface and that there are also free Golgi bodies (10).

Processes similar to those described have been observed in numerous exocrine glands (Bowen) in the biliary secretion (Cramer and Ludford, Solé and De Robertis) the enamel organ (Bowen) and so forth.

In some endocrine cells in which it is not possible to demonstrate any secretion products by cytological methods (parathyroid) the variations of the Golgi apparatus are illustrative of the functional state of the cells and permit us to demonstrate the existence of a true secretory cycle (see Chapter XI)

There have been attempts to explain the intervention of the Golgi apparatus in secretion on the basis of the properties of adsorption which it manifests toward various stains and other substances. Trypan blue injected into an animal seems to combine with the proteins of the plasma and, secondarily is fixed in the region of the Golgi apparatus (Bowen). The same thing happens with other acid stains mixed with flour (Kedrowsky). Also, there has been recognized the absorption of vitamin C, iron, copper compounds, gold and protargol, by the Golgi apparatus.

These results suggested that the Golgi apparatus might act in secretion as a condensation membrane for the concentration in drops or granules of products elaborated in other locations that diffuse through the cytoplasm. These products may be lipid, yolk, bile components, enzymes, hormones, and so forth. (Kirkman and Severinghaus). According to this theory the Golgi apparatus would act by surface action and would not intervene in synthesis or in the transformation of the products. Nevertheless, this action might not be purely passive and could have also a catalytic character. The same considerations which we made in regard to the chondriome could apply in this case. Neither can the idea be discarded that phenomena of synthesis are produced by enzymatic action in the interior of the Golgi apparatus.

In relation to this hypothesis it is interesting to point to the observation recently made that in the cells of the intestinal epithelium alkaline phosphatase is concentrated in the Golgi apparatus (Emmel). This seems to be the case also with the acid and alkaline phosphatase in several kinds of epithelial cells (Deane and Dempsey). These facts may indicate a participation of the Golgi apparatus in metabolic processes.

According to various authors, the presence of vitamin C in this organoid would also be related to these processes. According to Hirsch, when a cell is in process of elaborating a secretion product, the Golgi apparatus absorbs vitamin C. This is accumulated in the internal, osmiophobic part ("internum") which later increases in size and is transformed into the secretion product. According to other authors, the Golgi apparatus would have a protective action for vitamin C, preventing the rest of the cytoplasm from oxidizing it.

In summary. Although the existence of a relationship be-

tween secretion and Golgi apparatus seems to be established, the explanation of this relationship has not yet left the domain of hypothesis. If this is the present situation for the secretory cells, even more nebulous is the interpretation of the functional significance of the Golgi apparatus in the nonsecretory cells and particularly in the nerve cells, where it has such a considerable development. It has been thought that it may intervene in the secretion of fats, the elaboration of Nissl bodies, the metabolism of carbohydrates, and so on, but it is safer to affirm that, up to the present time, there is no satisfactory theory to explain in general form and for all cells the function of the Golgi apparatus.

### CELL CENTER

The cell center is a cytoplasmic organoid which is found in the majority of animal cells and in some cells of the lower plants. Its morphology is very different according to the functional state of the cell and, in this regard, one must distinguish the cell center in interphasic state from that which is found at the beginning of cell division.

In the *interphasic state* (between two mitoses) this organoid is generally constituted of a small spherical granule, the *centriole* which frequently is double (diplosome) (Fig. 46). In certain cases, the centrioles are multiple and in the giant cells of the bone marrow they may be very numerous. In some cells, the centrioles are elongated and have the form of a little rod. In the spermatozoa the form is even more complicated.

The *position* is, in general, fixed for each type of cell. In some cells, it has a tendency to occupy the geometrical center. This happens in ideal conditions in the case of the leucocytes, with a nucleus in the form of a horseshoe, or when the nuclear mass is small and is displaced but, in general, the centriole is pushed back by the nucleus and by the products elaborated by the cytoplasm. Nevertheless, even in these cases, the position is relatively fixed and axial, since if one draws a line between the center of the nucleus and the centriole, this coincides with the *axis* of the cell (Heidenhain). Such is the case in the cylindrical epithelial cells in which the centriole or centrioles occur in the central part of the apical end beneath the membrane.

The centrioles are not generally visible *in vivo*, but their constancy, characteristic position, coloration with acid stains, their affinity for iron hematoxylin and, particularly their behavior during mitosis, permit us to affirm their real existence in the cytoplasm.

The functional significance of the cell center will be studied along with cell division in Chapter VIII and its relation to cilia and flagella will be treated in Chapter XI.

In general it can be said that the cell center seems to be an organoid adapted to the mechanical manifestations of cellular energy.

## CHROMIDIAL SUBSTANCE

Under these and other denominations\* is included the basophilic material which is found in many cells and has staining reactions similar to the nuclear substance. In the higher animals it is found especially in nerve cells, in which it constitutes the Nissl bodies, and in the cells of serous secretion. The chromidial substance may be considered as a specialized part of the basic cytoplasm with a special chemical composition in which the nucleoproteins predominate.

In recent years, due to the employment of absorption microspectrography in the ultraviolet, a great advance has been made in the knowledge of the nature of this chromidial substance and of its possible role in cellular metabolism. For this reason, to facilitate its study, we shall include it in Chapter VII where, after an analysis of the chemical composition of the nucleus, we shall consider the problem of the cytoplasmic nucleoproteins, of which the chromidial substance is a particular case.

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## Chapter VI

### PLASMA MEMBRANE AND CELL PERMEABILITY

In several chapters of this book it has been shown that the cell constitutes a unit with a definite chemical organization and that this organization is, to some extent, maintained independent of the environment. For example, the ionic content of cells is generally relatively constant, even though it may be very different from that of the circulating blood (see Fig 5). This particular difference is maintained throughout the life of the cell probably by continuous control of the penetration and exit of molecules and ions exerted by a thin membrane surrounding the cytoplasm, called the *plasma membrane*. However other mechanisms of control involving parts or the whole protoplasm also take place and are particularly important in certain types of cells.

The property of this membrane of regulating the exchange between the cell and the medium is generally called *permeability*. The study of permeability is a fundamental branch of general physiology and obviously exceeds the scope of this book. Here some considerations about permeability processes will be made only when they bear an interest from the point of view of structure and function of the cell.

#### PLASMA MEMBRANE

In Chapter III the cell membrane was studied from the purely morphological point of view. It was said that the plasma membrane generally is invisible with the microscope, but that outside of it one may find other protective layers which reach to the limits of microscopic resolutions. Such is the case with the plant cells (Fig 118) which present a thick cellulose wall, covering and protecting the true plasma membrane. Animal cells may also be surrounded by cement like substances adsorbed on the periphery constituting visible cell walls. These protecting or adsorbed layers generally play no role in permeability.

Electron microscope studies of cultured cells show a definite boundary at the periphery of the cytoplasm (Fig 36) but do

For details on this subject the reader is referred to The Symposia of the Faraday Society 1937 and Cold Spring Harbor 1940. Davson and Danielli, 1943; Hoerber 1945.

not resolve a particular structure (Porter) The electron microscope study of red cell envelopes will be mentioned below

Although generally invisible, the existence of the plasma membrane cannot be disregarded, since it is demonstrated by various experiments with microdissection (see Chapter III) by the fact that not all substances can enter freely into the cell—a property of selectivity which is to some extent similar to that shown by artificial membranes—and, furthermore, because it can be demonstrated by polarization microscopy or by a special optical apparatus called the *leptoscope* (see below)

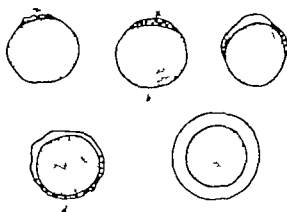


Fig. 47 Successive stages in the separation of the membrane of the egg of *Echinorachnus parma*. This process begins at the point of entrance of the spermatozoan, which is marked by an x (After Just)

In some special cases a cell membrane may readily become visible. Such is the case with the egg of the sea urchin in which, after the penetration of the sperm, a membrane is separated on the surface which prevents penetration of other spermatozoa into the fertilized egg (Fig 47) This, of course, does not exclude the possibility that there is formed another membrane invisible to the microscope, in contact with the protoplasm

### Structure of the Plasma Membrane: Theories

The study of the molecular organization of the plasma membrane has been considerably hampered by its extreme thinness. Recently it has been possible to make a direct approach to the problem by the development of new optical methods, but up to the present time results are still meager. Most of the data are of indirect nature and derive mainly from the study of the permeability properties of red cell membranes, marine eggs and muscle.

Theories of plasma membrane structure are generally based

on such indirect information. Thus, for example, the fact that the rapidity with which different molecules penetrate into the cell depends a great deal on their solubility in lipid solvents has led to the concept that the plasma membrane may contain a fine layer of lipid substance (Overton)

This theory is also favored by electrical measurements which indicate the presence of a high impedance at the plasma membrane (Cole and Curtis). Furthermore, data from chemical studies of the lipid content of the plasma membrane of erythrocytes, which when spread in a monolayer was considered to amount to a surface equivalent to twice the superficial area of the erythrocytes, led to the formulation of the theory that the plasma membrane is composed of a bimolecular layer of lipid molecules (Gorter and Grendell). However to explain the passage of water soluble molecules through the plasma membrane, a sort of sieve-like structure with a protein component has been claimed (see below)

Other indirect information came from the study of interfacial tension of different cells. While at the air-water interface there is a tension of 70 dynes per centimeter and at a water-oil interface about 10 to 15 dynes per centimeter the surface tension of cells is almost nil. By the use of centrifugal force upon sea urchin eggs to a point in which the elongated cell breaks into two halves (a process which can be watched with the centrifuge microscope see Chapter III) the surface tension can be calculated. This was found to be 0.2 dynes per centimeter in the unfertilized *Arbacia* egg (Harvey). Also by compressing eggs or other cells with a flat gold ribbon a very low surface tension (0.08 dyne per centimeter) was observed (Cole). Such a low tension is usually explained as due to the presence of protein layers upon the lipid components. When a very small amount of protein is added to a lipid-water model system the surface tension is lowered to comparable low figures.

To explain all these facts the theory was proposed that the membrane is constituted by a *lipid layer* of small molecules with a *protein layer* adhering at the lipid-aqueous interface (Danielli and Harvey).

As shown in Figure 48 the lipid layer is conceived as being bimolecular with the polar groups situated at the lipid-aqueous interface, while the nonpolar groups are adjacent to each other. The protein molecules disposed at both interfaces are arranged with their polypeptide chains in a direction perpendicular to the lipid molecules. This protein layer is thought to be composed by a sort of mesh of long molecules arranged in parallel lamellae.

at the interface and with nonpolar groups presenting to the lipid phase and polar groups directed toward the aqueous phase. The elasticity and the relative mechanical resistance which the plasma membrane possesses in spite of its delicacy are attributed to the presence of these layers of proteins, which are thought to maintain the cohesion of the different parts of the plasma membrane.

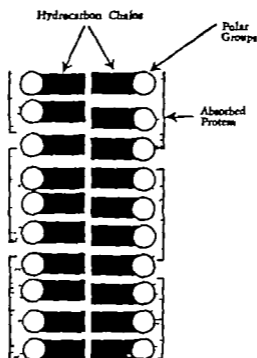


Fig. 48. Diagram of the molecular structure of the plasma membrane. (After Danielli.)

### Structure of the Envelope of the Erythrocyte

Direct information concerning structure of plasma membrane has been obtained in different cells by polarization microscopy (see Chapter IV). However in most of the cases the presence of the plasmagel (that is, the gelled layer of cytoplasm closely associated with the plasma membrane) which is much thicker has rendered very difficult this kind of structural analysis. This difficulty is avoided by the use of red cell envelopes. If mammalian erythrocytes are treated with a hypotonic solution, hemolysis occurs and hemoglobin and salts are removed, leaving a thin envelope called the ghost. Different data tend to indicate that these envelopes represent almost exclusively "plasma membranes" with very small internal cytoplasm. Although it is obvious that erythrocyte ghosts may not be typical plasma membranes, it is the only material so far isolated and studied by structural and chemical methods. Further research is necessary

to establish if data obtained on this material can be applied to the plasma membrane of other cells

**Chemical Composition.** Red cell envelopes can be obtained in amounts large enough for chemical analysis. Lipids in a concentration sufficient to account for a surface layer 40 Å thick were found (Gorter-Grendell). This lipid part is mainly composed of lecithin, cephalin and cholesterol (Parpart and Dziemian). An acidic protein called *stromatin* has been isolated (Jorpes) which is considered to represent a special type of protein, with a particular amino acid composition (Beach and collaborators, Balentine). The content of several amino acids is represented in Table VI and it may be noted that, irrespective of the phylogenetic differences, the content is fairly constant in different animals. The thickness corresponding to this protein component in the red cell envelope is approximately 70 Å (Waugh).

TABLE VI  
AMINO ACID CONTENTS OF LIPID EXTRACTED ERYTHROCYTE STROMA  
(After Beach, Erickson, Bernstein, Williams and Macy 1939)

	Histidin	Arginine	Lysine	Tyrosin	Tryptophane	Cystine	Methionin
Beef	1.0	3.1	3.3	2.0	1.2	0.87	1.3
Sheep	2.3	3.3	3.3	2.0	1.1	0.85	2.1
Horse	1.9	4.9	3.3	2.0	1.1	0.83	1.9
Hog	2.1	4.6	3.3	2.8	1.2	1.11	1.7
Man	2.1	4.8	3.5	2	1.2	0.90	1.8

**Polarization Microscopy.** With the use of refined polarization methods (see Chapter IV) indirect information can be obtained regarding the orientation of the protein or lipid constituents of the plasma membrane (Schmitt, Bear and Ponder). Erythrocyte ghosts show a faint negative polarization cross with a radial optical axis. This negative cross is enhanced by extraction with lipid solvents, indicating that it is due to fibrous proteins oriented in the plane of the envelope surface. Furthermore, by immersion of ghosts in glycerol or urea, which have a higher refractive index than water, the negative form birefringence disappears and is replaced by a positive cross with radial axis. This fact is an indication that lipid molecules are oriented radially.

Although polarization optics indicate the preferential orientation of both proteins and lipids in the envelope, they yield no information about the actual distribution of these two components in the plasma membrane.

*Electron Microscopy* Wolpers (1941) who studied the membrane of human erythrocytes with the aid of the electron microscope, reaches the conclusion that lipids and proteins are not arranged in layers, but as a lattice. Proteins form a kind of mechanical and dynamic framework which constitute a brush heap (micellar) structure. Between the meshes of this protein frame, the lipid component is distributed, filling holes existing in the protein layer. According to Wolpers, these lipid holes are responsible for some of the processes of permeability.

As an evidence of his theory Wolpers extracted the lipids with lipid solvents. The electron microscope photograph of such treated erythrocytes, in addition to a fibrous structure, shows a series of empty holes distributed where the lipid components of the membrane were probably situated. The distribution of the holes does not suggest the existence of molecular layers of proteins and lipids. If a lipid extracted ghost of an erythrocyte is stretched during the drying process (indispensable for the study of a preparation under the electron microscope) the fibrous nature of the protein component is very clearly visible.

*Leptoscope* To measure the thickness and to analyze the composition of the plasma membrane, an optical apparatus, called the leptoscope, has been devised. The idea of this apparatus is based upon the fact, described by Blodgett and Langmuir that the thickness of the film on a glass may be calculated from the intensity of light reflected by the film, if the refractive indices of the air, film and glass are known. Waugh and Schmitt adapted this idea for the study of thickness of the cell membrane (1940).

The principle of this method consists in comparing the intensity of light reflected by the plasma membrane (erythrocyte ghost) with the intensity of that reflected by molecular layers of barium stearate of known thickness. By spreading a fatty acid, such as stearic acid, on water under pressure, a monomolecular film can be obtained. In such a film, molecules tend to orient perpendicularly to the surface of the water with the polar groups ( $-\text{COOH}$ ) immersed in the water and the hydrocarbon chains sticking out toward the air. If a glass slide is dipped through this film into the water, a monomolecular film can be deposited with the polar groups attached to the glass. By dipping again, a second monomolecular film can be deposited on the slide which will have the polar groups oriented in the opposite direction (Fig. 49). By this technique a number of bimolecular layers, each one 48.8 Å in thickness, can be laid on the glass and also a steplike film can be built, each step having a thickness increment of 48.8 Å.

The leptoscope is composed of two microscopes, which receive, in reflected form, the same light intensity the field of which can be seen simultaneously with an ocular comparison. Under one

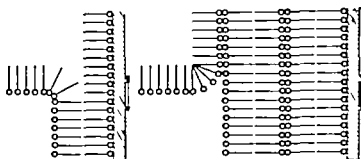


Fig 49 Diagram representing the building up of molecular films at an air water interface *Left* a glass slide previously coated with a monomolecular film of barium stearate (see the polar groups attached to the glass surface) is dipped in water having a monomolecular film at the interface. The second monomolecular layer attaches to the first by the nonpolar ends. *Right* several bimolecular layers of barium stearate have been deposited on the glass slide by successive round trips into and out of the water (Courtesy of D Waugh.)

microscope are placed hemolyzed and dehydrated erythrocytes (ghosts) and under the others the steplike layers of stearate. The intensities of reflected light are matched until the thickness of

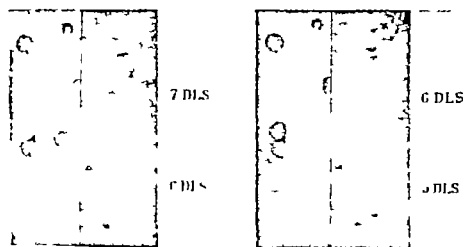


Fig 50 Photomicrographs taken with the leptoscope in which may be seen, in the same field, the stroma of hemolyzed corpuscles (dark discs situated at the left) and bimolecular layers of stearate of various thickness (at the right) (After Waugh, taken from Da son and Danielli.)

the latter reflect the same quantity of light as do the erythrocyte ghosts (Fig 50). Thus it was found that the thickness of the membrane corresponds to two to four molecules of stearate, or about 200 Å. This apparatus also permits an indirect determina

tion of the chemical composition of the membrane and establishes the relative thickness of the lipid and protein parts of the membrane. For instance, if fat solvents are applied so that only the protein component remains, the thickness of the membrane is reduced to a corresponding proportion. In erythrocytes of various animals, it was found that the thickness of the membrane ranges between 140 and 220 Å. In the rabbit it is about 215 Å, of which 110 Å belong to the protein layer and the rest to the lipid layer. This thickness of the lipid layer is equivalent to two bimolecular layers. Since the erythrocyte is only slightly permeable to water and to salts, it is quite possible that the plasma membranes of other cells are thinner i.e., they are composed of one layer of lipids only.

Considering the above mentioned dimensions, we have to bear in mind that the thickness of the membrane is related to a dry film only. Observations of ghosts in the way of drying tend to indicate that the red cell envelope contains less than 25 per cent water. This fact leads to its consideration as a desolvated structure in which the component molecules are in close association (Waugh).

Another interesting observation brought forth with the leptoscope is that at the central part of the ghost, which corresponds to the biconcave region, the thickness is frequently found to be 30 to 40 Å larger than at the periphery. This observation has been related to the typical biconcave shape of mammalian red cells and the process of reversible transformation of biconcave discs into spheres under appropriate conditions.

### OSMOTIC PRESSURE AND PLASMA MEMBRANE

The importance of osmotic pressure in the life of the organism is fundamental. In plant cells, osmotic pressure maintains the turgidity of the protoplasm. As was observed by De Vries, when these cells are placed in a solution which has an osmotic pressure similar to that of the intracellular fluid (isotonic solution) the cytoplasm remains adhered to the cellulose wall and is not changed. When the solution of the medium is more concentrated (hypertonic solution) the cell loses water and the cytoplasm retracts from the rigid cellulose wall. On the other hand, when the solution of the medium is less concentrated (hypotonic solution) the cell swells even to the point of bursting.

Since the plasma membrane of the cell is permeable to water and to certain solutes, the osmotic pressure is maintained by a mechanism which regulates the concentration of the dissolved substances in the interior of the cells. The passage of various

solutes across the membranes of animal cells does not occur with the same facility

Osmotic pressure also plays an important part in the formation of certain body fluids such as lymph and interstitial fluid.

At the end of the past century Hamburger demonstrated that osmotic pressure plays a very important role in the life of the cell. He found that the cell membrane behaves like an osmotic membrane and that a solution of 0.951 per cent of sodium chloride maintains the mammalian erythrocytes intact, while in less concentrated solutions, they are hemolyzed. In a medium of higher concentration, however the red corpuscles, due to loss of water show retraction. These experiments appear to be applicable to all animal cells both of a multicellular organism and of Protozoa (with a few exceptions, as among the Rhizopoda). On this basis, from the biological point of view one can group solutions into three classes (1) Isotonic solutions, which have an osmotic pressure the same as that of the cells. For example, 0.3 M solutions of nonelectrolytes are isotonic in relation to cells of mammals the same applies to the 0.9 per cent solution of sodium chloride. (2) Hypotonic solutions, which have an osmotic pressure less than that of the cells. For example, a 0.66 per cent solution of sodium chloride, which is isotonic for the erythrocytes of amphibia is hypotonic for the cells of mammals and may bring about the hemolysis of the red corpuscles (3) Hypertonic solutions, which have an osmotic pressure greater than that of the cells

From the examples mentioned above, it can be concluded that the tonicity of the medium is of great importance to cell function. This fact has given rise to the adoption of the physiological solutions, which have the same total osmotic pressure as the blood of the animals

In a higher organism, the regulation of the osmotic pressure of the body as a whole is carried out principally by the kidneys. In these, the hydrostatic pressure of the blood brings about the outward passage of water from the glomerulus (urine)

In the plant cells, there also are mechanisms for osmotic regulation. In general, the osmotic pressure in these cells is about 10 atmospheres (in *Penicillium* it reaches even as much as 100 atmospheres) and the permeability of their membranes, those of the vacuoles as well as the plasma membranes, undergoes permanent changes in order to regulate the intracellular osmotic pressure. To regulate their water balance, plants possess secretory glands which function as osmotic machines. Animal cells generally lack the turgidity which characterizes plant cells although there are exceptions, as in the case of *Tubularia*. On the other

hand, the unfertilized eggs of some marine animals, such as the sea urchin, behave like genuine osmometers. Since they are spherical, one can, by measuring the diameter determine the volume and the changes which this undergoes with changes in the osmotic pressure of the medium. In this material it can be demonstrated that the velocity of osmosis increases with temperature. Many bacteria behave in the same manner. This permits determination of their internal osmotic pressure by finding at what concentration their volume does not change (see Knaysi, 1944).

In many unicellular organisms the osmotic equilibrium is maintained by means of a contractile vacuole. It is this organoid which extracts the water from the protoplasm and contracts, eliminating its contents into the external medium. The unicellular organisms which lack contractile vacuoles regulate their excess of water by elimination across the cell membrane.

#### CELL PERMEABILITY

We may define permeability as the rate of movement of a substance through the permeable layer under a given driving force. S. C. Brooks and Mathilda M. Brooks, from whose book (1941) this definition is taken, stress that two factors have to be kept in mind (1) the characteristic of the cell (and nuclear) membrane for the passage of molecules and (2) the driving force of the latter which may be completely "independent of any property of the membrane. Permeability is of fundamental importance for the functioning of the living cell and the maintenance of satisfactory intracellular physiological conditions. This function determines which substances can enter the cell, many of which may be necessary to maintain its vital processes and the synthesis of living substances. It also regulates the outflow of excretory material and water as they are eliminated from the cells. Cell permeability undergoes continuous changes which depend upon the physiological state of the cell and various external conditions such as temperature.

#### Methods

Cell permeability can be studied by various methods. One frequent method involves observation of plasmolysis in plant cells, which is easily observed under the microscope. Excellent material for the study of permeability particularly of the permeability of water are the eggs of some marine animals (*Arbacia*, *Chaetopterus*, and the like) whose shape, under normal circumstances, is spheric and whose size is very constant. They swell or shrink, respectively in hypotonic and hypertonic solutions, with

out losing their spherical shape. This swelling and shrinkage can be determined by measuring the diameter of the egg and then calculating the volume

Another widely used method is that of the hemolysis of erythrocytes. There are various optical devices to measure the degree of hemolysis (by the change of intensity of color of the solution in which the red corpuscles are suspended) and this, related to the time factor gives a fairly accurate idea of the penetrability of a substance. These methods are mainly used for the study of nonelectrolytes

Lately some new methods were developed and of these the study of permeability with radioactive isotopes is particularly important. In this case, the tracer elements are used instead of normal compounds and their permeability through the membrane is measured directly by the Geiger counter. In order to measure the amount of substance which penetrated inside the cell during a definite period of time, this method is a great improvement of the method of direct chemical analysis of the cell contents. Whereas the latter method is used mainly to determine the permeability to nonelectrolytes, the isotopes are generally used to study the permeability to electrolytes

### Permeability to Molecules

Many soluble substances penetrate to the interior of the cells due to the fact that they can dissolve in the lipid layer of the plasma membrane. Nevertheless, the fact that the plasma membrane is crossed by molecules of water and by numerous compounds insoluble in fats leads to an assumption that the lipid layer may not be continuous and that it may contain spaces or pores which permit the passage of such substances. From this supposition it follows that, besides the factor of solubility cited above, there may intervene other factors such as the size of the traversing molecules and the size of the pores in the membrane. Collander and Bärklund (1933) demonstrated that the rapidity with which certain classes of molecules penetrate into the cell (*Chara*) depends directly on their solubility in lipids and on the size of their molecules: the more soluble they are, the more rapidly they penetrate. Among various substances which are equally soluble in lipids, those with the smallest molecules enter most rapidly (Fig 51)

### Permeability to Ions

The molecules of nonelectrolytes are generally stated to pass across membranes more readily than the electrically charged



in side II there will be found, besides the original number of ions ( $b$ ) an  $x$  number of ions coming from side I. At the same time on side I there is a lesser ( $x$ ) number of ions. This new situation can be represented as follows



Since the velocity of diffusion of  $\text{I} \rightarrow \text{II}$  is proportional to the concentration of  $\text{Na}$  and  $\text{Cl}^-$  in side I, this can be expressed as  $(a - x)^2$ . Simultaneously the speed of diffusion of  $\text{II} \rightarrow \text{I}$  is proportional to the product of the concentration of  $\text{Na}$  and  $\text{Cl}^-$  in side II, which may be expressed as  $(b + x)x$ . The ionic equilibrium is reached when the speeds of diffusion in both directions are the same, which can be expressed as follows

$$(a - x)^2 = (b + x)x$$

From this equation, which is called Donnan's fundamental equation, we may deduce: (1) that the concentration of the ions  $\text{Na}^+$  and  $\text{Cl}^-$  in I is the same (2) that the number of  $\text{Na}^+$  ions is greater in compartment II than in I (3) that the concentration of  $\text{Cl}^-$  is greater in I than in II and (4) that the concentration of  $\text{Na}^+$  in II is greater than that of  $\text{Cl}^-$

The Donnan equilibrium plays a role in biological phenomena and permits a partial explanation of the ionic equilibria established between the cells and the surrounding medium when there are diffusible and nondiffusible ions in the system. Proteins form such nondiffusible ions. It was on the basis of Donnan equilibrium that Van Slyke and collaborators were able to interpret the difference in the content of bicarbonate and chlorine between the erythrocyte and the serum, taking hemoglobin, which is present in high concentration in the erythrocyte, as the nondiffusible ion.

At the present time there exists little doubt that metallic ions, both anions and cations, enter and leave the cells. This interchange is probably active in cells and tissues in the process of growth since the concentration of salts remains constant even though the mass of the protoplasm is increasing. It also is very intense in various secretory cells such as those of the salivary and sudoriparous glands and especially those of the stomach, which lose a great quantity of salts that are replaced from the blood across the vascular membrane. Likewise, muscular and nervous tissues, during their physiological activity, show an active interchange of ions. The penetrability of the different metallic ions is variable. In the order of their penetrability we have

Rubidium > Potassium > Sodium > Lithium >  
Magnesium > Barium > Strontium > Calcium.

The penetrability expresses the relative speed, under standard conditions, with which various substances cross a particular plasma membrane. This is a property of the entering substance, while permeability is a property of the plasma membrane.

Study of the permeability to ions has advanced considerably during recent years due to the employment of radioactive isotopes, and though there might be a difference in the penetrability of a radioactive isotope as compared with the normal compound, the combination of various methods gives us, nevertheless, a fairly accurate idea of its penetrability.

In order to explain the rapid penetration of the cell by ions, without osmotic effects, Brooks suggested the existence of an ionic interchange across the membrane through electrically charged pores. According to this theory, there exist in the plasma membrane, in addition to noncharged pores, others with a positive or negative electrical charge. The positively charged pores attract the anions but repel the cations, and an opposite activity is carried on by the negatively charged pores. These pores are considered to be located principally in the protein layer of the membrane, which contains more water and would facilitate the movement of the ions. Furthermore, the sign of the charge would depend upon the proportion existing between the amino group (+) and the other basic groups, and carboxyl groups (-) of the amino acids adjacent to the pore. If there were a predominance of basic groups, the pore would be positive, or if the acid groups predominate, it would be negative. The existence of such charged pores, besides the Donnan equilibrium, would enable us to explain the selectivity which some cells possess for certain ions.

The problem of cell permeability is highly complex, and although some of its mechanisms are known, many others have not yet been elucidated. There is little doubt that besides the factors of solubility in lipids, penetration through pores without or with an electrical charge, and ionic interchange, there must exist other mechanisms which, under certain conditions, permit the entrance of large molecules. The same phenomenon of diffusion cannot, in many cases, be explained exclusively on the basis of the laws of permeability and of molecular size. It is known, for example, that glucose diffuses into the cells with great ease while other sugars do so with difficulty. This is probably due to the fact that, since glucose plays an important role in the general metabolism of the body, it is utilized as soon as it enters the cell. Under these conditions, in order to maintain the equilibrium, large amounts of glucose must penetrate inside the cell. The other sugars, such as saccharose, are metabolized very slowly and, therefore, when equilibrium is reached they cannot diffuse any more into the cells. In these examples it will be noted that there exists an intimate relation between permeability and the metabolism of the cell.

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## Chapter VII

### STRUCTURE AND CHEMICAL COMPOSITION OF THE NUCLEUS IN THE INTERPHASIC STATE

Since the discovery of the nucleus as a constant part of the protoplast (Brown, 1835), the majority of cytologists have been interested in the extraordinary phenomena which it manifests during mitotic or kinetic division of the cell and in meiosis or the division of maturation of the germ cells. As a consequence there was considerable progress in karyology which is a branch of cytology dealing with the nucleus or karyosome, to the detriment of the study of the cytoplasm or cytosome.

The methods of fixation and staining and of vital observation made it possible to demonstrate that the nucleus passes through a series of complex but remarkably regular and constant changes which are characterized by the disappearance of the karyotheca or nuclear membrane and the appearance of intensely staining bodies the *chromosomes* (Gr. *Chroma* color *Soma*, body). This is so characteristic that it led to a definition of the nucleus as "any formation surrounded by cytoplasm from which chromosomes arise during division" (Belar).

From this point of view every cell passes, in the course of its life, through two periods: one being *interphasic* or *metabolic* and the other being *mitotic* or a period of *division*. Both are characterized principally by changes in the nuclear structure. In the interphasic or metabolic period the karyosome is found in the usual state of nondivision. \* On the other hand, in the period of mitotic division the whole group of changes occurs which leads to the division of the chromosomes and the reconstitution of the daughter nuclei.

In this chapter we shall study the structure and the chemical composition of the interphasic nucleus, leaving for the following chapters the analysis of the mitotic process and the heterotypic mitosis or meiosis of the germ cells. Finally in Chapter XI we shall consider the nucleus in its cytogenetic aspect, that is to say as a true *organ of heredity* the bearer of the *genes* or *factors* determining the hereditary characteristics.

This period is spoken of commonly as one of *repose* but this designation is not appropriate, because many functions are being carried on at this time (Sharp).

### Morphology of the Nucleus (Constancy Form, Size, Number Position)

In all cells of higher animals and plants there is found a nucleus with the general characteristics described in Chapter III. On the other hand, in certain lower organisms, such a nucleus is not apparent. In some Flagellata and Infusoria the nucleus is represented by granules of nuclear substance (chromatin) scattered through the cytoplasm. In bacteria no nuclear structure

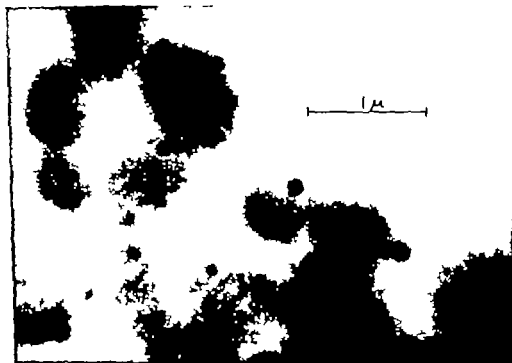


Fig. 52. Electron micrograph of *Sarcina flava*. Bacteria show polygonal form because of mutual compression. Cell "walls" and dense bodies, whose nuclear nature is still under discussion, are clearly seen.  $\times 25,000$ , 110 kv (Courtesy of Miss W. Van Iterson, Institute for Electron Microscopy Delft, Netherlands.)

is distinguishable by ordinary methods but, at times, various workers have interpreted as the equivalent of nuclei scattered granules or a diffuse uniform material which has the microchemical characteristics of nuclear substance. Recently with the electron microscope, it has been possible to show in bacteria a body with the characteristics of a nucleus (Fig. 52).

On the other hand, in viruses, chemical analysis demonstrates that they contain nucleoproteins, substances which are also essential components of the nucleus. Modern studies tend to demonstrate the constant presence of the nucleus or its equivalent, in cells and the importance of the nucleoproteins in all of the manifestations of life.

The *form* of the nucleus may have some relation to that of the cell or it may be completely irregular. In the first case, if the cell is isodiametric (spherical, cubical, polyhedral) the nucleus is generally spherical. On the other hand, in cylindrical cells or prismatic or fusiform cells, it tends to be ellipsoidal and in squamous cells, flattened. Examples of irregular nuclei are found in the leucocytes (nucleus in the form of a horseshoe or with many lobes) certain Infusoria (moniliform) glandular cells of many insects (branched), spermatozoa (elliptical, pyriform, lanceolate, and so forth, according to the species)

The *size* of the nucleus is variable, but in general, it bears some relation to that of the cytoplasm. This may be expressed numerically in the so-called *nucleoplasmic index* (NP) (R. Hertwig)

$$NP = \frac{V_n}{V_c - V_n}$$

$V_n$  being the nuclear volume and  $V_c$  the volume of the cell.

This NP index states that there is a relationship between the volume of the cytoplasm and that of the nucleus, of such a nature that when the former increases, the second also should increase. The lack of maintenance of the NP ratio would seem to act as a stimulus to cell division. In general, the younger cells have more voluminous nuclei, but this rule is not constant.

Almost all cells are *mononucleate* but there exist also *binucleate* cells (some hepatic and cartilage cells) and *polynucleate* cells. In these last, the nuclei may be very numerous (up to one hundred in the case of the polykaryocytes (or osteoclasts) of bone marrow (Fig 141) In the syncytia, which are large protoplasmic masses not subdivided into cellular territories, the nuclei may be extremely numerous. Such is the case with the striated muscle fiber and with certain syphonal algae and the like, in which the nuclei may number several hundred.

The *position* of the nucleus is variable but, in general, characteristic for each type of cell. In embryonic cells it almost always occupies the geometric center but it commonly becomes displaced as differentiation advances and as specific parts or reserve substances are formed in the cytoplasm. In these cases, the changes in position almost always appear to be passive. Thus, in the adipose cells, or in eggs rich in yolk, the nucleus is forced towards the periphery by the accumulation of paraplast. In glandular cells it is located in the basal position, the granules occupying the apical cytoplasm. Whatever may be the position which the nucleus takes up in differentiated cells, it almost always

is surrounded by a zone of cytoplasm which maintains its undifferentiated embryonic aspects. In some rare cases, the displacement of the nucleus may be related to the nutrition of the cell (root hairs)

### Structure of the Nucleus

In Chapter III, in describing the microscopic structure of the nucleus in the living cell, we said that, with some exceptions, vital or supravital observation reveals only the presence of a nuclear membrane or karyotheca and of one or more nucleoli (Fig 12). On the other hand, in fixed and stained material the structure of the nucleus is distinguished by its great complexity and varies according to the type of cell and the fixative used. In general, there can be distinguished (Fig 53 A) (1) a nuclear membrane or kary



Fig 53 Interphasic nuclei of pancreatic cells. Fixation by the freezing-drying process. A, Azan staining the nucleolus (in red) the chromonemic filaments with their enlarged portions (chromocenters) and the nuclear sap are seen. B Feulgen's reaction the nucleolus gives a negative reaction in the nuclear sap the reaction is slightly positive. C Action of ribonuclease and staining with Azan. The nucleolus does not stain, due to the digestion of the ribonucleic acid. (De Robertis, Montes de Oca and Raffaele.)

otheca which appears in optical section as a line well defined, both on the cytoplasmic side and on the nuclear (2) an unstained or lightly acidophilic mass, the *laryolymph* or *nuclear sap* which fills completely the nuclear space in which are found the other components (3) flakes or twisted filaments which contain chromatin, a substance with characteristic staining properties and which are distributed throughout the nuclear sap and are united by a fine lightly staining reticulum, the *linin*. In general, chromatin stains with basic stains (basichromatin) but some flakes may be acidophilic (oxychromatin) (4) flakes of chromatin of coarser nature situated among the smaller ones, the *chromo-*

*centers* or *karyosomes* also called nucleomic nucleoli or false nucleoli. The significance of these bodies, as well as that of the other chromatin granules, will be studied below (5) spherical bodies or nucleoli often of considerable size (nerve cells, ovocytes, and so forth) and either single or multiple, which resemble the karyosomes, but differ by their staining affinity which is, in general, acidophilic.

The structures described may present great variations in the different cells of an animal, and even in the same type of cell in different species. The chromatin is the component which shows the greatest variation. In extreme cases, it may appear in fine granules, giving the nucleus a dusty aspect, or in very large granules which stain intensely. In some cases, the chromatin is deposited on the internal surface of the karyotheca even to the point of forming a continuous layer beneath it (chromatic membrane).

Another characteristic of the structure of the interphasic nucleus is its great variability under the action of different fixatives. The same material may present diverse aspects according to the fixative employed. Thus, for example, with osmic acid the nuclear content is almost homogeneous, only the nucleoli being distinguished. With mercuric chloride the chromatin is very coarse and the linin reticulum well defined. With Flemming's mixture (osmic acid, chromic acid and acetic acid) the chromatin is less coarse and the aspect of the nucleus corresponds more to that mentioned above.

*Significance of the Nuclear Structure* The variations which are observed in the appearance of the chromatin, under the action of different fixatives, and, above all, the apparent lack of nuclear structure in living cells and the results of micromanipulation (page 48) caused many authors to question the significance of the appearance of the fixed nucleus. According to this interpretation, the interphasic nucleus, with the exception of the nucleoli, would be actually homogeneous and the chromatin reticulum would result as an artifact of fixation. This opinion is reinforced by considerations on the colloidal nature of the nuclear components. The chromatin would be, according to some authors, diffuse and would correspond to the dispersed phase of the nuclear colloid. Thus, due to its great instability, might be separated out from the dispersing phase by the action of the fixative and precipitate in finer or coarser flakes (Della Valle). Other authors admit the existence of two distinct phases: the *karyolymph*, which is considered as a colloid sufficiently stable to be precipitated only with acids and fixatives, and the *karyotin* a more labile and com

plex colloid dispersed in the karyolymph, which would precipitate with great facility through mechanical or weak chemical action (Strugger). The facts cited justify the opinion that one should be cautious and, up to a certain degree, skeptical, as regards the presence of aggregates of chromatin in the interphasic nucleus, but they do not justify drastic and extreme conclusions because the evidences in this regard are, for the most part, negative (Bensley).

The optical homogeneity showed by the living nucleus does not imply the existence of a structural homogeneity since hydrophilic colloids, with a wide layer of hydration, may not be visible even in the darkfield, and, on the other hand, many appear when their hydrophilia diminishes or when they precipitate. Some authors have described in certain living cells the existence of a heterogeneity characterized by a denser portion in the form of granules or of a reticulum (Chapter III). On the other hand, in nuclei apparently homogeneous, it is possible to bring about the appearance of a heterogeneity by mechanical action (micromanipulation) by asphyxia, circulatory alteration, action of various substances, and so on, and this modification is reversible within certain limits (van Herwerden). Furthermore, with vital stain one can demonstrate the existence of preformed structures in deed, if cells of the intestinal epithelium of the frog are placed in an atmosphere of hydrogen, the nuclei stain with neutral red and show heterogeneity (Nassonow). Definite results have been obtained applying the method of fixation by freezing and drying, by means of which all the inconveniences of chemical fixation are avoided. In no case was there found a homogeneous distribution; it was proved, on the other hand, that the nuclear structure is similar to that which is demonstrated by the best cytological methods of fixation (Fig. 53).

All these facts permit us to affirm that, although the living nucleus may be optically homogeneous, this does not signify that there is a structural homogeneity. This structure, although it may be somewhat altered by the fixatives, actually exists and is characterized by the segregation of the chromatin in special regions of the nuclear space. Nevertheless, the image of fixed nuclei should not be accepted without a critical attitude because, besides the true structures, there are others, such as the *linin*, which seem to be the result of a protein precipitation.

The structure of the interphasic nucleus has considerable interest from the cytogenetic point of view. The modern interpretation of the mechanism of heredity is based on the idea that the *chromosomes* which appear at the time of division, are

autonomous and that they are *continued* through the interphasic nucleus and the succeeding divisions. The chromosomes would be the bearers of the genes or hereditary factors in the different cellular generations and for this reason, even in the interphasic nucleus, are regarded as permanent structures, preserving a certain individuality. Modern cytological studies, particularly on plant cells, permit us to affirm that the autonomy and continuity of the chromosomes actually exist. (See Chapter VIII.)

The chromosomes are composed of two fundamental parts: the *chromonema* and the *matrix* (see Chapter VIII). The former is a twisted chromatic thread which forms a kind of structural member for the chromosome. The second is a substance which impregnates the chromonema and which, at certain moments of division, hides the chromonema. The chromonema is the only part of the chromosome which persists in the interphasic nucleus since the matrix appears only during division (Fig. 53 A).

According to this interpretation, the chromatin filaments or chromonemata which are found in the interphasic nucleus (see Chapter VIII) represent, in this state, the chromosomes. Furthermore, the chromocenters or karyosomes, also called false nucleoli or chromatin nucleoli, are more condensed zones (heterochromatic) which have retained part of the matrix of the chromosome. In some cells there exists a single chromocenter for each chromosome and, in this particular case, one may determine the number of chromosomes by counting the karyosomes of the interphasic nucleus. In general, the counting of the chromosomes can be done only during cell division.

The *submicroscopic structure* of the nucleus is described in Chapter IV.

### Physicochemical Properties of the Nucleus

The *specific gravity* of the nucleus is, in general, greater than that of the basic cytoplasm. Of the different nuclear structures, the one with the greatest specific gravity is the nucleolus which, in some eggs, is sedimentated directly by the action of gravity. With ultracentrifugation the nucleus is deformed and the nuclear structures are stratified in the following layers (from the centrifugal to the centripetal pole): (1) nucleolus, (2) basichromatin, (3) oxychromatin, (4) karyolymph (Beams).

The *viscosity* of the nucleus is variable. In some cases it seems to be greater than that of the cytoplasm, in others, scarcely twice that of water. Like the fundamental cytoplasm, the content of the nucleus possesses "structural viscosity" that is, it reacts like a *thixotropic fluid* capable of reversible solation and gelation. This

property can be recognized especially with micromanipulation the mechanical agitation of the microneedle is sufficient to produce localized gelations in the nucleus

The nuclear pH determined by micromanipulation, is more alkaline than that of the cytoplasm (7.6 to 7.8) (Chambers) on the other hand, the buffering power is very small.

We saw in Chapter III that one of the characteristic properties of the cytoplasm is that of forming a new membrane (in the presence of the  $\text{Ca}^{++}$  ion) when the superficial cytoplasm is ruptured. This property does not exist in the nucleus, so that when the nuclear membrane is broken the karyolymph flows out and the nucleus collapses without showing any tendency to repair. This different behavior may be due to the differences which exist in the electrical charge. The cell membrane generally has a *negative charge*, while the *karyotheca* has a *positive charge* for which reason it could not combine with cations such as the  $\text{Ca}^{++}$  ions. If we grant that the appearance of a new film is dependent upon the formation of a calcium proteinate, this would explain why the nuclear membrane lacks the property of repair (Churney).

In contrast to the nuclear membrane, the *chromosomes* and *nucleoli* react in the electric field in a distinct manner and appear to have a *negative charge*.

The nuclear chromatin, from the electrochemical point of view is an ampholyte, having at the same time both positive and negative charges, and the behavior of the molecule as a whole with respect to the external charge depends upon the pH of the medium. At certain pH's it behaves as a cation (+) and is displaced in the electric field (electrophoresis) toward the negative pole on the other hand, at other pH's it acts as an anion (-) and goes toward the positive pole. There exists a definite pH at which both charges are equal and the protein is not displaced toward either pole. This is called the *isoelectric point of the proteins* (IP) (see Chapter II).

The *isoelectric point of the chromatin* was determined by the staining of fixed structures with acid and basic stains at different pH's. It was found that when the pH is varied the chromatin is stained with the acid as well as with basic stains, and that the isoelectric point varies between pH 3 and 5 (Pischinger). It is interesting to recall that the IP of the nucleic acid is 2 and that of histone 8.5. Recently it was possible to determine the isoelectric point of the nucleus by electrophoresis. Nuclei of the cells of the salivary glands of *Drosophila* showed an IP at pH 3.3 to 3.6 for the chromosomes and at pH 10.6 to 11.6 for the membrane. The IP of such a high value shown by the karyotheca makes us think that this structure may be constituted particularly by basic proteins, that is, those which contain many basic amino acids, such as histidine, lysine and arginine (Churney).

### Chemical Composition of the Nucleus

A great part of our present knowledge of the chemical composition of the nucleus comes from the classical chemical studies of Meischer (1869) and Kossel (1891) on cells of pus, spermatozoa of fishes, hemolyzed erythrocytes of birds and on leucocytes and cells of the thymus and liver submitted to peptic digestion. In

recent years, it has been possible to confirm these results and to make new advances, due to the development of methods for the isolation of nuclei (Fig. 54) and for the extraction of nucleoproteins.

All these studies indicate that the most important components of the nucleus may be *nucleoproteins* which are conjugated proteins resulting from the combination of *nucleic acid* with *simple proteins*. In the spermatozoa of the trout, the nucleoproteins constitute more than 96 per cent of the solid material and the



Fig. 54 Isolated nuclei of hepatic cells. (Courtesy of A. L. Dounce.)

isolated nuclei of erythrocytes contain almost 100 per cent (Mirsky and Pollister). The protein part is composed essentially of two strongly basic protein types: protamines and histones. Recently it has been possible to isolate another protein component, not well identified, which contains sulfur (Mayer and Gulick). Protamine seems to be found only in the spermatozoa of some fishes; on the other hand, histones have been found in all the other cells which have been analyzed. Histones constitute quantitatively an important part of the nucleus; for example, in the erythrocytes of birds they represent 40 per cent of the dry weight of the nuclear material. From these results it follows that the *nucleohistones* appear to be very important components of the nucleus and, in particular, of the chromatin.

Recently doubt has been cast on the idea that the protein part of the nucleus is exclusively histone and it even has been

affirmed that the principal component of the nucleus is an acid protein, called *chromosomin* which would constitute from 50 to 72.6 per cent of the nucleoproteins (Stedman and Stedman) this, however remains to be confirmed.

According to Mirsky and Pollister the nucleus contains a complex nucleoprotein which they call *chromosin*, which contains three components desoxyribonucleic acid, histone and nonhistone protein. The nucleic acid is in highly polymerized condition, is very viscous when dissolved, and fibrous when precipitated. Histone contains only traces of tryptophane, while the nonhistone protein contains about 1 per cent of this amino acid.

*Nucleic acid* combines with a protein in a saltlike union which is not very strong and may be broken with relative ease. It has three components phosphoric acid, carbohydrates and purine and pyrimidine nitrogenous bases. According to the theory of Levene, nucleic acids appear to exist as tetranucleotides, that is, in aggregates of four *nucleotides* each of which results from the combination of phosphoric acid with a carbohydrate residue and a nitrogenous base (see Chapter II).

Two principal classes of nucleic acid have been described, differing essentially in the type of carbohydrate. Thus, (1) the *thymonucleic acid* or *desoxyribonucleic acid* or *desoxypentose nucleic acid* is found in nuclei and contains desoxyribose, and (2) *ribonucleic acid* or *pentosenucleic acid*, isolated originally from yeasts, contains the carbohydrate *ribose*. The other components (phosphoric acid, purine and pyrimidine bases) are almost similar in the two types of nucleic acid.

The purine bases are adenine and guanine and the pyrimidines, cytosine, thymine and uracil. In Figure 55 we indicate the form in which these nitrogenous bases are combined with the carbohydrate and the phosphoric acid to constitute the nucleotides. It can be appreciated, furthermore, that the desoxyribonucleic and ribonucleic acids differ from each other not only with respect to the pentose, but also in their pyrimidine base contents. Thymine is found in the former but is replaced by uracil in the latter. This last difference, nevertheless, has no histochemical expression.

The available evidence from the literature seems to support the theory of the tetranucleotide structure first proposed by Levene (Greenstein). According to this, the four different nucleotide residues are bound together by phosphate-ester linkages (Fig. 56). However isolated desoxyribonucleic acids are very highly polymerized and the molecular weight ranges between 500 000 and 1 000 000 or more. Since the molecular weight of a tetranucleotide is about 1400 a molecule of such a nucleic acid may contain 500 to 1000 tetranucleotides. The molecular weight of the ribonucleic acid is generally much lower.

Sodium salts of desoxyribonucleic acid form fibers with intense negative birefringence and in aqueous solution show strong negative streaming birefringence and structural viscosity. Electron microscope studies of nucleic acid show long branching and

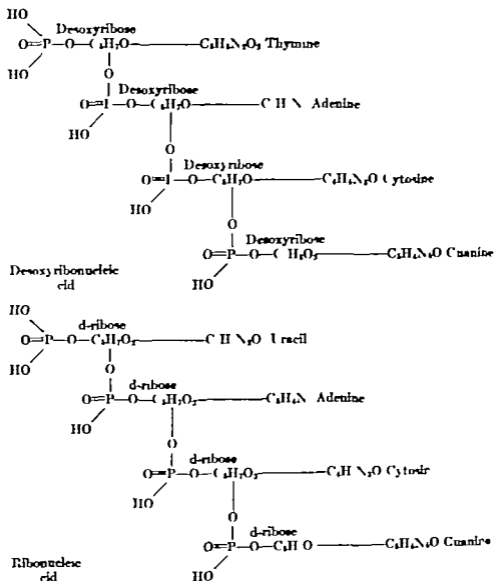


Fig. 55 Chemical formulas of desoxyribonucleic acid (thymonucleic) and of ribonucleic acid.

anastomosing fibrous processes (Scott). All these properties indicate a considerable molecular asymmetry. This molecular asymmetry and the tendency of the nucleic acid particles to become oriented under stretching are also revealed by the dichroism shown in the ultraviolet light (Caspersson). With ultraviolet polarized light it was found that the absorption at 2600 Å is higher for light polarized with its electrical vector perpendicular to the stretching, and lower for light with its vector parallel.

*Nuclear Reaction of Feulgen* —Thymonucleic acid takes part in the composition of the nuclear nucleohistones, but modern studies have demonstrated that ribonucleic acid is also found in some structures of the nucleus and constitutes an important material in the cytoplasm of many cells (see below) From the histochemical point of view the topography of thymonucleic acid

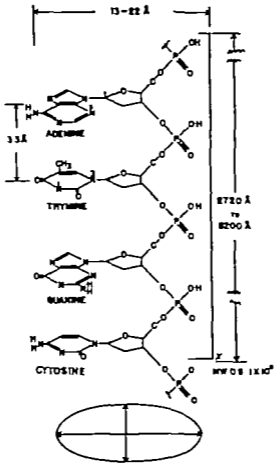


Fig. 56. Most generally accepted stereochemical formula of desoxyribonucleic acid. This molecule is thought of as a column of nucleotides in which the purine and pyrimidine bases as well as the sugar rings are coplanar and perpendicular to the phosphate chain. The published data from the physicochemical studies are summarized and the range of lengths, widths and spacings between the rings is indicated. The ellipse below represents the negative uniaxial birefringence and dichroism of the nucleic acid. (Courtesy of J. Scott.)

can be studied by means of the *nuclear reaction of Feulgen*. This method consists in carrying out an acid hydrolysis and then treating the tissue with Schiff's reagent (fuchsin decolorized with sulfur dioxide, leucofuchsin). The parts which contain thymonucleic acid give a *positive* reaction (recolor the fuchsin) because of the presence of the desoxyribose. On the other hand, this reaction is completely *negative* for ribonucleic acid.

Applied to cells, the Feulgen reaction is positive in the nucleus

and negative in the cytoplasm (Fig 53 B) In the former, the chromatin of the chromonema and the chromocenters gives an intense reaction, while that in the nucleolus is negative. After fixation by freezing and drying a slight positive reaction has been seen in the karyolymph (Bensley)

Another modern method for the histochemical study of nucleic acid is that of the *absorption spectrophotometry in the ultraviolet*. This has been applied to cells and tissues by Caspersson, and has given important results

*Absorption Microspectrophotometry in the Ultraviolet* Various organic substances may possess a characteristic absorption spectrum which may manifest itself in a particular spectral region, as in visible light or in the infra red or ultraviolet. Thus, for example, if a solution of hemoglobin is traversed by white light it shows a specific absorption band at 5590 Å. This property of absorption which, when it occurs in the visible spectrum, determines the coloration of various substances, depends upon molecular, atomic or electronic resonances In organic compounds such resonances are often present on a definite part of the molecule called the chromophore, which is ordinarily a chemical group possessing unsaturated bonds

Most organic substances composing the cell do not absorb visible light and, for this reason, are colorless. On the other hand, some of them, such as nucleic acid, have a typical absorption in the ultraviolet spectrum (Fig 63 B) The curves which are obtained with the spectrophotometer are characteristic and aid in the identification and localization of such a substance where it is encountered Furthermore, the height of the curve is proportional to the concentration so that quantitative measurements can be made. Absorption microspectrophotometry thus carries out one of the aims of histochemistry, since it permits not only a qualitative study but likewise a quantitative study of certain cellular components Another of the great advantages of this method is its high degree of sensitivity According to Caspersson, quantities of nucleic acid of  $10^{-11}$  mg can be determined in an area of  $1 \mu^2$  of a section

The necessary apparatus consists of (1) a *source of light* which gives forth a spectrum rich in ultraviolet radiation (such as a mercury arc) (2) a *monochromator* or a set of filters which separates the light emitted into distinct spectral bands and which permits a narrow monochromatic band to pass through the optical system of (3) a *microscope with optical system made of quartz*, or of other dielectric material permeable to the ultraviolet by which the preparation is studied, generally unstained

and (4) a system to measure the absorption (Fig 57) The measurements can be carried out of the desired wavelengths by means of a *photoelectric cell* or by a *photographic* method using calibrated plates In this case, the absorption curve is obtained

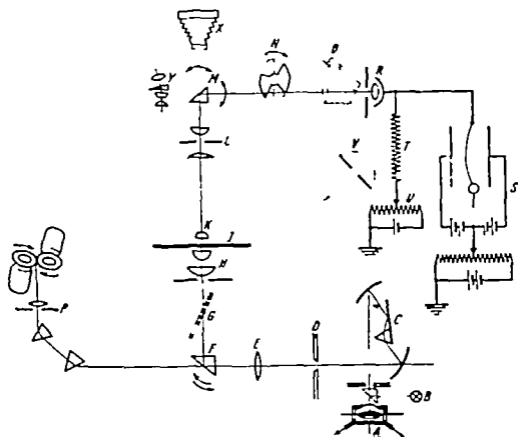


Fig 57 Apparatus for absorption macrospectrophotometry of Caspersson. A mercury lamp B tungsten band lamp C monochromator D second monochromator slit E, lens F movable 90° quartz prism G quartz plate (used with photocell I to compensate for changes in the lamp) H condenser I object K objective L, ocular with adjustable diaphragm M accurately movable prism of molten quartz N rotating sector O telescope for centering P Köhler's rotating spark gap arrangement R, photocell S electrometer T leakage resistance U four step potentiometer X camera Y Köhler focuser for the ultraviolet, interchangeable with prism M (From Caspersson)

by measuring the degree of darkening of the plate with a densitometer

The image of a section in ultraviolet light varies with the wavelength employed With wavelengths close to the visible spectrum (3600 to 3000 Å) there is very little absorption and cellular structures are not distinguishable If shorter radiations (3000 to 2300 Å) are used, differential absorption is apparent and reaches a variable maximum according to the chemical composition of structures in the material Thus, for example, protein

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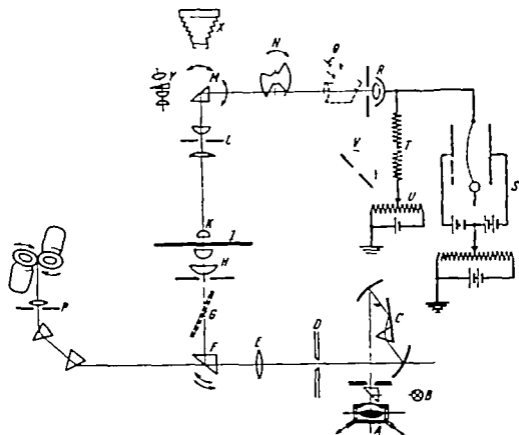


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structures give a maximum absorption at 2800 Å (Fig 61) while nucleic acids show a characteristic peak at 2600 Å (Fig 62)

The specific absorption of nucleic acid (2600 Å) is due to the presence in the molecule of purine and pyrimidine bases and, for this reason, is similar in both thymonucleic and ribonucleic acid. This fact, especially, has a particular interest because, as will be recalled, the nuclear Feulgen reaction is positive only for the thymonucleic acid. The two histochemical methods complement each other. Microspectrophotometry permits the localization of the two types of nucleic acids without distinguishing between



Fig 58 Ultraviolet photomicrograph in the band 2560 Å. Interphasic nucleus and chromosomes in a preparation from the testis of the locust.

them, while the nuclear reaction of Feulgen shows the presence of thymonucleic acid. By comparing the results from each method, the distribution of the ribonucleic acid can be determined; this is very important in the study of the chemical composition of the nucleolus and the *cytoplasmic localizations of the nucleoproteins*.

With this method it has been proved that nucleic acid has a wide distribution in nuclear structures and that it exists not only in the basichromatin, but also in the oxychromatin and in the nucleolus (Fig 58). The content of nucleic acid varies in the different functional stages of the cells of serous secretions. This may indicate that this substance takes part in these metabolic processes. During cell division, the nucleic acid is localized exclusively in the chromosomes and increases in the prophase of mitosis before the reduplication of the genes in the chromosomes (Chapter VIII), later diminishing at the termination of the division. This fact would indicate that the nucleic acid plays an essential role in cell division.

In addition, changes in the content of nucleic acid are reflected with microspectrophotometry.

photometric technique concerning the nature of the protein of the nucleus. The more complex protein decreases as the amount of histone increases and reaches a maximum at metaphase, decreasing in telophase. However, studies of histones extracted from nuclei tend to contradict these results as the absorption spectrum of the histone did not differ from that of a higher protein (like albumin) (Mirsky).

A complementary method for the study of the chemical composition of the nucleus is the employment of enzymes which specifically digest certain nuclear components. If *proteolytic enzymes* (pepsin, trypsin) are allowed to act, the nuclear structures lose their individuality and fuse together but the nucleic acid persists (Caspersson). On the other hand, if *nucleotidase* is allowed to act, the nucleic acid disappears, but the protein part of the chromosome persists (Mazia). These studies seem to demonstrate that the chromosomes possess a continuous protein skeleton upon which their structural integrity depends.

The content in lipids of the nucleus is far below that of the cytoplasm.

The distribution of minerals can be studied by means of *microincineration*. In the spodogram it is seen that the nucleus has a greater concentration of ash than the cytoplasm, the ash being composed of phosphorus, potassium, sodium and particularly calcium and magnesium. These ashes are found, apparently, in greater proportion in the chromatic structures. Recently it has been possible to show the localization of calcium and magnesium in the nucleus by means of the emission electron microscope (Scott).

### Chemical Composition and Function of the Nucleolus

As was said above, the nucleolus gives a negative response to Feulgen's reaction, which indicates a lack of thymonucleic acid (Fig 53 B). Its chemical composition was clarified by means of the absorption microspectrophotometry in the ultraviolet. The nucleolus shows a typical curve with an absorption maximum at 2600 Å (nucleic acid) and another at 2800 Å (protein). These two findings demonstrated that the nucleolus contains ribonucleic acid, probably in the form of nucleoprotein (Caspersson and Schultz). Similar conclusions were reached using ribonuclease, an enzyme which digests ribonucleic acid (Brachet, Gersh, De Robertis, Raffaele and Montes de Oca). However, the nucleolus is generally surrounded by a ring of Feulgen positive chromatin (Fig 53 C).

In a series of experiments, the chemical composition of the

nucleolus was studied in relation to genetic control (Caspersson and Schultz) It was found that either rearrangements in the heterochromatic regions of the chromosomes in *Drosophila* or sex differences produced changes in the proportion of protein to nucleic acid and in the type of protein of the nucleolus.

The *function of the nucleolus* and particularly the significance of the cyclic changes which it shows in mitosis, have eluded cytological interpretation. It has been known since early times that the nucleoli of the interphasic nucleus disappear at the beginning of cell division (prophase), at the same time that the chromosomes increase their content of chromatin, and that, at the end of division (telophase) the nucleoli reappear at the same time that the chromatin diminishes. The relation which exists between the nucleolar cycle and the chromosomic cycle has been clarified in part by the demonstration, in plant cells, that the nucleoli are in intimate relationship with certain chromosomes. Each nucleolus lies in contact with a chromosome and thus possesses at the point of union a special region called the *organizer of the nucleolus* (see Chapter VIII). The nuclear material is derived (in the telophase) from all of the chromosomes present, but it is accumulated and organized only in the region of the organizer of the nucleolus. This region of the chromosome, which is *heterochromatic* (Chapter VIII), would be bound up with the chemical cycle of the ribonucleic acid of the nucleolus just as the other heterochromatic zones of the chromosomes would be related to the synthesis of thymonucleic acid and to the content of ribonucleic acid of the cytoplasm (See below)

#### Cytoplasmic Localizations of Nucleic Acid and Chemical Composition of the Chromidial Substance

For some time it has been suspected that there may be an interchange between the nucleus and the cytoplasm, especially characterized by the passage of nuclear substances to the cytoplasm. Subsequently, studies carried out by means of the Feulgen reaction appeared to negate completely the existence of such an interchange, since, while this reaction is positive in the nuclear chromatin, it is negative in the nucleolus and in the cytoplasm (Fig 53 B)

However the presence of ribonucleic acid in cytoplasm was demonstrated first in plant cells by separating the nuclei from the cytoplasm (Behrens). It was found that the cytoplasm contains ribonucleic acid while the nucleus contains thymonucleic acid. More recently it has been possible to show that cells in active growth contain ribonucleic acid in the cytoplasm (Cas-

persson and Schultz) Thus, at the tip of the root of the garlic plant, the cytoplasm of the cells, in regions showing numerous mitotic figures, has a curve of absorption with a typical maximum



Fig. 59 Absorption spectra of the cytoplasm of liver cells of the chick embryo 1 three-day embryo 2 and 3 six-day embryo 4 and 5 newly hatched chick. (From Caspersson and Thorell.)

at 2600 Å, while this is lacking in the cells at the base of the root (where no mitoses exist) In the oocytes of the sea urchin the cytoplasm next to the nucleus has the absorption characteristic of nucleic acid, but this is lacking in the peripheral cytoplasm. This fact brings up again the problem of the extrusion of nucleoli

from the nucleus. This *nucleolar* extrusion has been described in the ovocytes of different species by various authors. In the teleosts, the ovocytes have, at a particular time in development, numerous nucleoli which adhere to the karyotheca and, in more advanced stages, bodies with the characteristics of the nucleoli are found not only within the nucleus but also in the perinuclear cyto-

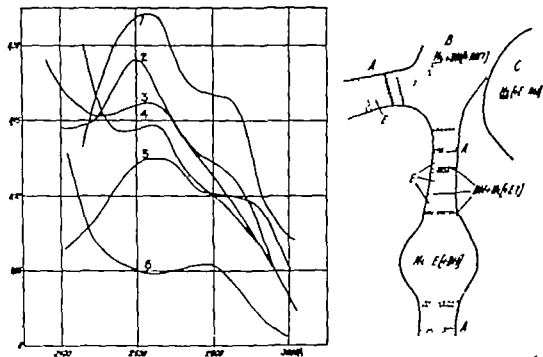


Fig 60 *Left*, Absorption curves of different chromosomic structures. Curve 1 chromocenter; 2 chromosome attachment to chromocenter; 3 euchromatin; 4 heterochromatin; 5 chromocenter; 6 interband. (From Caspersson.)

*Right*, Diagram of the structure of the salivary gland nucleus. A, euchromatin; B heterochromatin; C nucleolus; DN desoxyribonucleotides; RN ribonucleotides; E, protein of the higher type (coagulable); H, proteins rich in hexose bases. Under lining indicates large quantities; brackets, small quantities. (From Caspersson.)

plasm. This nucleolar extrusion has been also studied in isolated germinal vesicles of amphibian eggs (Duryee). The presence of ribonucleic acid in the cytoplasm could be due, in part, to the passing out of nucleoli, but this would be an exceptional type of mechanism. The synthesis of ribonucleic acid could be carried out directly in the cytoplasm next to the nuclear membrane (Caspersson and Schultz). This nucleic acid would serve as a reserve for the building of the chromosomes in the segmentation of the egg.

On the other hand, in cells of the chick embryo it has been found that the content of ribonucleic acid is greater in the earlier stages of development, as it is in yeast cells in active division (Caspersson and Thorell). In Figure 59 it is clearly seen that

the content of ribonucleotides of the cytoplasm, as measured by the peak at 2600 Å, decreases continuously with the time of development and reaches a minimum in the newly hatched chick. In Figure 59 other differences in the shapes of the curves are seen, which the authors attribute to differences in the proportion of histone-like to coagulable proteins which have a lower concentration in hexone bases (histidine, lysine, arginine)

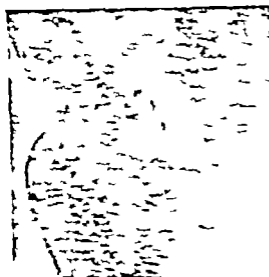


Fig. 61



Fig. 62

Fig. 61 Ultraviolet photomicrograph of pancreatic acini frozen and dried, taken at 2803 Å; the granules of zymogen are distinguished by their greater degree of absorption.

Fig. 62. The same acini as in Fig. 61 in the band 2650 Å, the nuclei and the chromidial substance are distinguished by their greater degree of absorption. (De Robertis, Nuñez and Del Conte.)

According to Caspersson, the longer wave maxima (beyond 2800 Å) are characteristic of the histone type of protein, in which the hexone bases predominate. The shorter wave maxima (below 2800 Å) correspond to more complex proteins. All these facts seem to indicate that the ribonucleic acid of the cytoplasm, like that of the nucleolus, plays an important role in the metabolism of the nucleus during the stages of division and of protoplasmic growth.

*Chromidial or basophilic substance* is found especially in the cytoplasm of nerve cells and in cells of serous secretion, and is characterized by its intense coloration with nuclear stains. In both cases, this substance is invisible in the living cell, but assumes definite forms after fixation (Nissl bodies, basal laminae, and so forth). Although numerous authors since the time of Hertwig (1899) have thought that it is chemically similar to nuclear

substance and even supported the idea of its nuclear origin, histochemical analysis could not demonstrate this to be true, because of a lack of adequate methods. Recently with absorption microspectrophotometry, it has been found that both the Nissl bodies of the nerve cells (Caspersson, Gersh and Bodian) and the basal substance of serous cells (Caspersson and colleagues) contain nucleoproteins of the ribonucleic acid type.

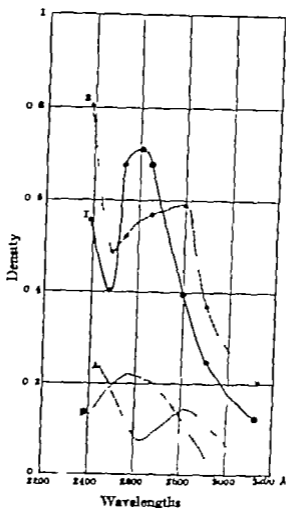


Fig 63. Ultraviolet absorption curves in pancreatic cells 1 curve of the basal cytoplasm (chromidial substance); 2 absorption curve of the apical cytoplasm (zymogen granules) A, absorption curve of albumin-globulin; B absorption curve of nucleic acid

In the case of the exocrine pancreas, which we shall take as an example, the basal part of the cell contains a homogeneous substance, the *chromidial substance* which stains intensely with toluidine blue (Fig 64) while in the apical part there are found zymogen granules. Applying the Feulgen method to this tissue, the reaction proved to be positive in the nucleus, but completely negative in the cytoplasm, a finding which demonstrates the absence of thymonucleic acid in the cytoplasm.

If an unstained section mounted in glycerin is observed with the ultraviolet microscope, it is seen that the absorption varies with the wavelength employed and with the part of the cell under consideration. Thus, the apical region which contains

the zymogen granules absorb especially at 2803 Å (Fig. 61) and at 2399 Å. On the other hand, the basal region shows its maximum absorption at 2650 Å (Fig. 62). The absorption curves of the base of the cell show a typical maximum in the neighborhood of 2600 Å with a high extinction coefficient (Fig. 63, 1). This maximum coincides exactly with that which is given by nucleic acid (Fig. 63, 2) and thus demonstrates the existence of a high concentration of nucleotides in this part of the cell. The curve corresponding to the zone of the granules differs (Fig. 63, 2) showing general characteristics similar to that of a simple protein (Fig. 63, 4). This signifies that the composition of the zymogen is essentially protein in nature.

Enzymatic methods of histochemical analysis have likewise demonstrated ribonucleic acid in the Nissl bodies (Gersh and



Fig. 64

Fig. 64 Pancreatic acini frozen and dried and stained with toluidine blue. The chromidial substance appears intensely stained.



Fig. 65

Fig. 65 Same as Fig. 64 but after digestion with ribonuclease the chromidial substance has disappeared.

Bodian) and in the basophilic substance of secretory cells. Thus, for example, if pancreatic cells are treated with ribonuclease, an enzyme which hydrolyzes ribonucleic acid, the chromidial substance disappears completely and can no longer be stained by basic dyes (Figs. 64 and 65).

Similar studies have been carried out on a series of cell types with ultraviolet absorption (Caspersson and colleagues) and the use of basophilic stains associated with the action of ribonuclease (Brachet). It has been seen that in many cells where the cytoplasm is intensely basophilic such as in the parotid gland, the

chief cells of the stomach, the basal layers of the skin, the crypts of Lieberkuhn, the hair follicles, the regenerative buds of animals and plants, there is a considerable quantity of ribonucleic acid. Plasma cells and lymphocytes to which have been attributed the elaboration of some of the globulins of blood plasma also contain a considerable quantity of cytoplasmic ribonucleic acid (Bing, Fagracus and Thorell White and Daugherty) In the lymphocytic series, it is noted that in the large lymphocytes there is a high concentration of ribonucleotides in the cytoplasm and at the same time a large nucleolus with a negative Feulgen reaction on the other hand, the small lymphocytes lack a nucleolus and are poor in cytoplasmic nucleotides. Similar results are found in the cells of the myelocytic and erythroblastic series (Thorell)

The same conclusion, namely that the ribonucleoproteins are relatively frequent components of the cytoplasm, has been reached by a completely different line of investigation. Chemical analysis of microsomes, isolated by ultracentrifugation from various cells, shows that they contain ribonucleic acid, and that there is a large quantity of this compound in most of the cells which are reproducing actively such as those of embryos and of tumors, and in the glands which have abundant basophilic material (Claude)

Modern studies show that the distribution and importance of the nucleoproteins in biology are much greater than had been supposed earlier. These compounds are found in all organic structures which, like the chromosomes and viruses, are capable of autoduplication, and their presence in the cytoplasmic microsomes leads some to suppose that these likewise might have the power to reproduce themselves. According to these concepts, the nucleic acid of the cytoplasm has an active part in the synthetic phenomena characterizing cell division and protoplasmic growth, and in the elaboration of proteins on the part of gland cells.

These facts, as well as others which will be mentioned in Chapter VIII, have been interpreted by Caspersson as supporting a general theory which relates protein synthesis with metabolism of nucleic acid in all living organisms. According to this theory the nucleus and, in particular the chromosomes, are considered as the center of protein synthesis. In the diagram (Fig. 60) the chemical composition of a prophasic chromosome is shown as it is found in a salivary gland of *Chironomus* (see Giant Chromosomes, Chapter VIII). It is seen that the euchromatin which constitutes the bands contains thymonucleic acid associated with histones, while the clear disks interposed between the bands lack nucleic acid and are formed of more complex proteins comparable to the globulins. On the other hand, the heterochromatin (which

in interphasic nuclei constitutes the chromocenters) contains the two types of nucleic acid and, in addition, great quantities of histone, while the nucleolus contains ribonucleic acid exclusively with a greater or less proportion of histones. The absorption curves of these different structures are shown in Figure 60. During the mitotic cycle there are produced, according to Caspersson, the following changes. In the prophase, the thymonucleic acid is deposited on the chromomeres, which are the bearers of the genes, so that in the metaphase all the nuclear material is accumulated in the strongly spiral chromonema. At the same time, the amount of protein is considerably reduced. In this phase, the quantity of thymonucleic acid reaches its maximum and that of ribonucleic acid its minimum. The telophase is characterized by a synthesis of material necessary for the duplication of the genes under the combined action of the hetero- and euchromatin and, simultaneously the thymonucleic acid diminishes. The euchromatin would produce higher proteins (coagulable) while the heterochromatin would produce proteins rich in hexone bases (histones). At this time, in intimate relation with the heterochromatin one or more nucleoli are developed which have similar characteristics. On leaving the nucleoli the histones diffuse toward the nuclear membrane, where they provoke the formation of cytoplasmic ribonucleoproteins about the membrane as they come into contact with the ribonucleic acid of the cytoplasm. The ribonucleoproteins of the cytoplasm in turn provoke the synthesis of the higher cytoplasmic proteins. In other words, the nucleoli and the cytoplasmic nucleic acids serve as intermediaries in the chain of processes which go from heterochromatin to the proteins of the cytoplasm.

In summary, this theory holds that in the processes of synthesis of intracellular proteins, there exists a close interrelationship between the nucleic acids situated in the chromosomes, the nucleolus and the cytoplasm.

Furthermore, there is evidence of a true metabolic interrelationship between the two types of nucleic acid found in the cells. Brachet postulates that the ribonucleic acid, which is localized in the cytoplasm and in the nucleolus, may be transformed into desoxyribonucleic acid of the chromosomes, and vice versa. Thus between these three zones of the cell (cytoplasm, nucleolus and chromosome) there would be a continuous metabolic interchange. The control and regulation of this interchange is thought to be localized in the heterochromatic zones of the chromosomes, which, in contrast to the euchromatic zones, are the parts which retain their content of nucleic acid in the interphasic stage (see Chapter VIII). (For enzymes of the nuclei, see Chapter X.)

## Functional Significance of the Interphasic Nucleus

All cells in synthetic activity have a nucleus and its lack is incompatible with a long and metabolic active life.

In the interphasic state, the nucleus is separated from the cytoplasm by a membrane which presents special characteristics of permeability and forms with the cytoplasm, a heterogeneous system in dynamic equilibrium. In these conditions, the nucleus, like the cytoplasm, is incapable of an autonomous existence, a fact which demonstrates that the interrelationship between them is necessary for the maintenance of a normal metabolism. Numerous experiments with fragments of cells without a nucleus have given proof that although such fragments can carry out some functions, such as those of reacting to stimuli and ingesting food (amebae) forming a cellulose membrane (plant cells), moving cilia (ciliated cells), in general they survive only a short time and are incapable of growth and reproduction.

On the other hand, if denucleated pieces of the eggs of echinoderms and amphibians, which are condemned to a rapid regression, are fertilized by spermatozoa of the same or other species, not only do they maintain their vitality but they can even divide and form perfect larvae (G Hertwig Boveri, Baltzer) Furthermore, the nuclei themselves cannot live isolated as they need a certain quantity of cytoplasm (Lillie)

These facts demonstrate that between the cytoplasm and the nucleus there must exist continuous interchanges of substances which permit the maintenance of the equilibrium of cellular functions and the normal synthesis of the protoplast.

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## Chapter VIII

### CHROMOSOMES AND CELL DIVISION

Of all the cell components, the chromosomes have been most thoroughly investigated. Their presence was demonstrated long before they were named *chromosomes* by Waldeyer in 1888. Forty years earlier, the botanist, Hofmeister, while studying the pollen mother cells of *Tradescantia*, portrayed them in drawings taken directly from living cells. This was the first concrete representation of these nuclear components to appear in biological literature.

A chromosome may be considered as a body endowed with a special organization, individuality and functional qualities, it is capable of reproducing its physical and chemical structure through successive cell divisions and of maintaining its morphological and physiological properties.

The great interest resides in chromosomes because of their important role in variation, heredity, mutation and evolution and in their control of morphogenesis, multiplication and equilibrium of vital processes. The surprising progress which has been made in recent years in the study of the behavior and structure of chromosomes has brought us face to face with numerous problems which signify a true revolution within the biological sciences.\*

Analysis of the organization and mechanism of chromosomes is of great importance because they contain the genes; these are independent units now considered to be the basic material governing the intrinsic mechanism of the organism.

It is a general belief that the chromosomes are observable only during the stages of cell division, in which they appear as little rods which stain intensely. However, in reality these elements are permanent entities of the nucleus in all the stages of cell life. Their appearance depends on the physiological state in which the cell is found and for this reason they may seem to adopt different aspects. Sometimes they are found as extremely

In spite of this, it is surprising—as White says (1942)—that the great advances made by cytogeneticists in the last decade have not been valued sufficiently by the biologists who work in other fields of investigation. This is particularly lamentable in those who are investigating the problem of cancer in which this subject has a fundamental importance.

delicate, slender and tortuous filaments in the interior of the nucleus, while in other circumstances, such as in the middle phases of mitosis (metaphase and anaphase) they appear as compact cylinders of characteristic form and size ✓

## MORPHOLOGY OF THE CHROMOSOMES

The external aspects presented by the chromosomes at certain stages of the mitotic or meiotic process may be studied by techniques involving fixation, with the minimum of altering artifacts which may change their normal aspect, form, or size. Such treatments accentuate the morphological characteristics without modifying them. The most propitious time to observe the morphology of the chromosomes is during the stages in which they are found to be most compact (metaphase and anaphase of cell division). It is here that one finds them as cylindrical bodies of quite solid consistency, that stain intensely with basic dyes (hematoxylin, safranin, crystal violet, orcein, carmine, and the like) ✓

The most appropriate animal tissues for the study of chromosomes are the sex glands, especially those of the male, the tail of the tadpoles of urodeles (Triton, axolotl) the salivary glands, esophagus and intestine, malpighian tubules and other organs of the larvae or nymphs of insects. In plants one can use the meristems of the root and stem, buds of young leaves, pollen mother cells and the embryo sac, and the microspores (pollen grains) in division. One may fix, embed, section and stain the tissues appropriately or more simply crush or smear a small piece of tissue on a slide and macerate it in a drop of reagent which fixes and stains simultaneously in a few minutes (acetic-orcein, aceto-carmine, lacmoid, and so on). By this second method one may obtain entire cells with their whole complex of chromosomes. One may also compress the smear between coverglass and slide, thus breaking the nuclear membrane and displacing the chromosomes outside of the cell, where they can be studied individually.

✓ In profile, during the anaphase, or seen from the poles, the chromosomes may be observed in three different forms (Fig 66) a straight rod or *telocentric* a J or *subtelocentric*, with two unequal arms, or a V or *metacentric* with two equal arms. As the form of a given chromosome is constant in all the cells of an individual or even within one species or genus, it may be useful in identifying specific chromosomes of the complex.

**Centromere** (Gr *Meros* part) At the point where the arms of the chromosome are joined, there is observed a constriction called the *primary* or *centric constriction* (Figs 66 67 and 68) in the middle of this is found a clear zone containing at times, a small granule or *spherule*. This clear region constitutes the *centromere* (also called the *kinetochore*) and is found in intimate functional relation to the movements of the chromosome during cell division. For some time, it was described as the point of in

section of the fiber of the achromatic spindle. Darlington (1936) as well as Schrader (1936), noted the similarity of the centromere



Fig. 66. The three morphological types of chromosomes according to the position of the centromere (indicated by a clear circle) *a*, telocentric; *b* subtelocentric; *c*, metacentric. The two first types are also called acrocentric.

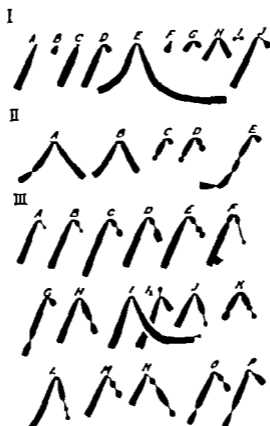


Fig. 67 Somatic chromosomes during the metaphase (in polar view) showing their different forms. In this diagram the chromosomes have been placed so that the centromere is found at the point where each letter occurs. *I* Chromosomes with a constriction corresponding to the location of the centromere. *II* Chromosomes with short secondary constrictions. *III* Chromosomes with long secondary constrictions. (After Darlington, 1937)

to the centriole. These two bodies are similar with respect to their behavior during the mitotic cycle, their appearance in living cells and their reaction with stains. The spherule has a diameter of

about  $0.2\ \mu$ , and the centromere about  $3\ \mu$  to  $5\ \mu$ , depending on the species

✓ The centromere delimits the arms of the chromosome, and its constancy of position determines the form of the chromosome (Figs 66 and 67) ✓

*Secondary Constrictions* Another of the morphological characteristics peculiar to the chromosome are the *secondary constrictions*. Likewise constant in their position and extent they

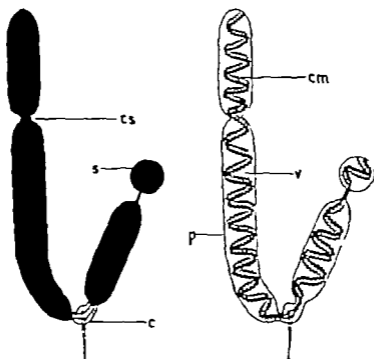


Fig 68. Schematic representation of the morphology and internal structure of a chromosome. *Left*, morphology of chromosome in the compact state, as it is observed by means of current techniques in metaphase or anaphase of mitosis. *Right*, internal structure of the same chromosome treated by a special technique during the anaphase. *cs*, secondary constriction; *c*, centromere; *s* satellite *cm*, chromonema in a double spiral, showing the major spiral and minor spiral *p* pellicle *v* sheath or matrix.

possess great value for the identification of particular chromosomes in a complex. They may be either *short* or *long*. They are distributed along the chromosome and are distinguishable from the primary constriction by the absence of marked angular deviation of the segments of the chromosome on either side (Fig 67).

*Telomeres* (Gr *Tele* far *Meros* part) This term is applied to each of the extremities of the chromosome which, as genetic investigations in *Drosophila* have demonstrated, present specific properties. If x rays or ultraviolet rays are allowed to act on the cells of *Drosophila*, the chromosomes undergo various fractures

The resulting segments may unite and different types of rearrangements among the fragments may occur, but the reuniting pieces do not fuse to the *telomere*, in any case. It seems as if the telomeres have a polarity which prevents other segments from joining with them, in contrast to the pieces obtained by breaking, which are bipolar and may rearrange themselves in variable forms (see Chapter IX). Chromosomes may even form a ring, if they have lost both telomeres.

### Satellites

Another morphological element which certain chromosomes present is the *satellite* or *trabant* (Navashin, 1912). The satellite is a rounded or elongated prominence separated from the body of the chromosome by a delicate chromatic filament. The size of the satellites is variable and their diameter may be the same as that of the chromosome or much smaller down to a point where they are almost imperceptible. Likewise, the filament of union may be long or short (Fig. 67). In well fixed chromosomes the satellite is always clearly distinguished. It is customary to designate as SAT-chromosomes those which have a satellite. The satellite and the filament are always constant in their form and size for each particular chromosome.

### Nucleolar Zone

Among the longitudinal differentiations of the chromosomes there are structures which resemble the secondary constrictions in that they interrupt the continuity of the matrix by a constriction. Such constrictions are called the *nucleolar zone*, the nucleolar organizer or the SAT zone. These constrictions are closely related to the formation of the nucleoli, although morphologically they are not easily distinguished from the ordinary secondary constrictions. Generally there are in each cell two chromosomes called nucleolar chromosomes, which have this special characteristic (Figs. 69 and 78). In nucleolar chromosomes the formative regions may also be found in the part where a satellite is united to the chromosome.

When Heitz discovered, in 1931, that during the telophase each nucleolus has its origin in a chromosome with a satellite, and that its formation takes place in the narrower region where the filament is found, he called this filiform part the SAT filament, which signifies in abbreviated form, "*sans acido thymonucleico*" (without thymonucleic acid). Heitz supposed that the filament did not stain because it lacks this type of nucleic acid. Later however it was demonstrated that it gives a positive Feulgen's reaction. By a happy coincidence the three letters SAT constitute an abbreviated expression for satellite, for which reason the designation of SAT chromosomes has been retained for those which possess satellites, and SAT-zones for regions which form nucleoli.

At the present time, it is thought that the region which produces the nucleolus is composed of the following elements (Figs. 69 and 78)

1 *Nucleolar organizer* This is the part of the chromosomes where the nucleolus originates, the region situated at the end of the chromosomes, where the filament joining the satellite emerges

2 *Nucleolar body* This is the part of the nucleolus where its development begins prior to the appearance of the nucleolus itself. This region is Feulgen negative.



Fig. 69 Nucleolar chromosomes of *Larum*. S secondary or nucleolar constriction. P primary constriction or centromere. (After Stewart and Bamford, 1943.)

3 *Filament of the satellite* This is a continuation of the chromonema without the matrix. It is a permanent formation of the chromosome, but may vary in length. In case a nucleolus is not formed the filament is retracted, whereupon the satellite becomes directly united to the chromosome.

The fact that the nucleolar regions are poor in chromatic substance (coming from the matrix) and therefore similar to the heterochromatic segments, has led to belief in the existence of a relationship between the heterochromatin and the composition of the nucleolus (see below)

### Structure of the Chromosome

The chromosome presents a very different aspect when its intimate constitution is studied and when one considers its internal or molecular organization. To study the internal structure of the chromosome, the classical techniques are of limited value,

and it has proved useful to apply the analysis by biophysical and biochemical methods (x ray diffraction, polarization microscopy, electron microscopy, protein chemistry, enzymology, ultraviolet microspectrophotometry, and so forth), and by experimental genetics and cytogenetics

When one studies unfixed chromosomes, or chromosomes fixed in some special way (such as by hot water, the vapors of nitric acid, hydrochloric acid or acetic acid, or dilute solutions of ammonia, sodium hydroxide or the like), one can observe a filament which is coiled into a spiral along the length of the



Fig. 70. *a*, Spiral structure in the chromosomes of *Tradescantia congesta*, during the first meiotic division. The arrow indicates the minor spiral. *b* Prophase of a pollen grain (microspore) of *Trillium grandiflorum*, showing the large turns of the spiral which are remaining from the time of their origin in the cycle of the previous division simultaneously a new cycle of spiralization has been initiated which is shown in the small turn of each one of the chromatids. (*a*, after Coleman and Hillary 1941 *b* after Sparrow 1942)

chromosome. This filament, which has a diameter in the neighborhood of the limit of resolution of the light microscope, has received the name of *chromonema*, a term created by Wilson in 1896, and applied to this structure by Vojdovsky in 1912

The methods mentioned above attack the outer portion of the chromosome, called the *matrix*, which disappears, leaving uncovered the helicoidal structure of the chromonema

In 1880, Baranetsky observed the chromonema in the pollen mother cells of *Tradescantia* but, at that early date, the interpretation of the structure was primitive. Although this structure has been studied principally in plants such as *Tradescantia* and *Trillium*, it may be seen also in Protozoa, certain insects, amphibia and so forth, in which the structure of the chromosomes is even more accessible for study. It is generally thought that the chromosomes of all organisms have a helicoidal organization.

In anaphasic chromosomes one may see a spiral chromonema coiled into close turns, like a spring in maximum longitudinal contraction (Figs 68 and 70). Such a coil shows two classes of spirals: the *minor* which has from 80 to 100 turns or more, and the *major* superimposed upon this, with larger turns numbering 10 to 30 (Fig 68). Each turn of the major spiral includes several turns of the minor. The number of turns depends upon the diameter and length of the chromosome, being inversely proportional to the former and directly proportional to the latter. If  $l$  represents the length of the metaphasic or anaphasic chromosome and  $d$  its diameter, the number of turns is equal to  $2l/d$ .

Darlington believes in the existence of a molecular spiral which acts on the internal mechanics of the chromonema calling forth the formation of the visible spiral, which reaches its maximum development during the metaphase. The number of filaments which compose the chromonema is a matter of discussion at the present time. During the metaphase, it may be single, double, quadruple or multiple, depending upon the material. The internal structure of chromosomes is depicted in Figures 68 and 79. The external part or limiting membrane is called the *pellicle* (Fig 68). The matrix forms the solid mass of the chromosome, acting like a filling substance which envelops the chromonema, covering it even up to the point of concealing it during the stages of maximum condensation of the chromosome. The *matrix* is also called the *sheath*, *hyalolemma* (Gr *Hyalos* crystalline *Lemma*, sheath) or *calymma*. The *chromomeres* (Gr *Chroma*, color *Meros*, part) are granules distributed along the length of the filiform chromosome, similar to the beads of a rosary. They are clearly observed in the prophasic stages, especially in meiosis, and appear like flakes of the chromosomal filament produced by accumulations of the chromatic substance. It is also postulated that these granules can be caused by the superimposition of smaller turns of the spiral, which would produce optical images resembling the thickenings in question.

The direction of the turns of the spiral is not always the same in a particular chromosome. The torsion may be reversed from one region to another. In this connection two interpretations of the processes of spiralization have been proposed. In one it is suggested that the individual genes have an internal spiral arrangement which determines the formation of the spirals observed with the microscope (see Chapter IV). The other interpretation suggests that the elongated and parallel fibers composing the chromosome may tend to form a spiral when they undergo a linear contraction. Therefore the direction of the helix is not constant in a particular chromosome or segment, since it may change at random. This apparently capricious mechanism tends to explain the observation that in each mitosis a new spiral is formed, as Whitt has demonstrated in certain species of Orthoptera (locusts). In an analysis of sixty-four spermatogonial cells, 11 descendants of a single cell, he

has seen that the spiralization is directed indiscriminately either toward the left or toward the right.

It is probable that the direction of the spiral is a phenomenon imposed upon the chromosome by conditions in the outside medium, either by the matrix itself or by contact relations between homologous or daughter filaments. The fact that it changes at random might indicate that probably it is not controlled by its own intrinsic molecular structure.

The genetic importance of the chromosome lies, as might be supposed, in the chromonema, since it is the physiologically active part. Cell division, whether mitotic or meiotic, is, in reality the history of the chromonema in the various aspects of its cycle of spiralization. (See below)

Of course, some of the fundamental facts concerning the biology of the chromonemata are the mechanism of their attraction at the time of conjugation their relations with crossing over and the later separation by unfolding to give rise to another similar chromonema

### GIANT CHROMOSOMES

Balbani in 1881 first observed these chromosomes in the salivary glands of the larvae of the fly *Chironomus*. In these glands there are giant nuclei, within each of which occurs a coarse strand resembling a rolled up ball of yarn. This represents the chromosomes. Heitz and Bauer in 1933 and Painter in 1934 discovered the extraordinary internal organization of these chromosomes, as seen in the salivary glands of the flies *Bibio* and *Drosophila* respectively.

For some time these elements were known as salivary chromosomes, but their designation ought to be changed to that of *polytenic chromosomes*, since it is considered most probable at the present time that these elements are the product of a series of successive longitudinal divisions of a common chromonema until they constitute a massive and coarse element, similar to a rope, characteristic of these giant chromosomes. Furthermore, it is not only in the salivary glands that these chromosomes are found, for they occur in other organs and tissues such as the esophagus, intestine, tubules of Malpighi and nerve cells, of the dipterous insects and of some Neuroptera, probably occurring also in other groups of insects which up to now have not been investigated by adequate techniques.

The technique for their study is extremely simple. The glands are crushed between the coverglass and the slide in a drop of a fixing and staining fluid (aceto-carmine or acetic-orcin) and the giant nuclei are broken open, leaving the chromosomes free to

spread out, facilitating the examination. The giant nuclei with their chromosomes and nucleolus may be observed even in the living state. The nuclei have a volume of about  $60\,000\text{ cu }\mu$ .

Micrurgical studies of fresh polytenic chromosomes have shown that they are soft, deformable bodies which when isolated can be stretched up to twenty five times the original length without breaking. They regain their former length when the stretching does not exceed tenfold (D'Angelo).

The fixed polytenic chromosomes appear as coarse strands of variable length (Fig. 71). In *Drosophila* the X or I chromosome reaches a length of more than  $400\text{ }\mu$ , some 250 times longer than the ordinary somatic chromosome of the same



Fig. 71 Polytenic chromosomes of *Drosophila melanogaster*. *a* View of the chromosomes of one nucleus, showing the dark bands and the clear interbands. Preparation by crushing in aceto-carmum. *b* Part of the sex chromosome X, as it is observed with ultraviolet light. Photograph taken at  $2570\text{ }\text{\AA}$ , which is the spectral band of maximum absorption for nucleic acid. (*b* After Schultz, 1941)

animal. In Figure 72, the difference in size existing between Chromosome IV of the same fly in an ordinary mitosis (indicated by the arrow) and in the salivary gland may be appreciated. The somatic number of chromosomes characteristic of the *Chironomus* is eight, but when the polytenic nucleus of a larva is observed at the end of its development, there appear at first to be only four chromosomes. This is because the chromosomes are found closely united in pairs in such a way that they appear as if there were four chromosomes. Similarly in *Drosophila*, instead of finding eight chromosomes, which is the typical number, only six coarse strands appear in polytenic nuclei (Fig. 73, 1 and 2) just as in the former example it was shown that the chromosomes form pairs even more closely united than in *Chironomus*, as if we were dealing with a true synaptic conjugation (Chapter IV). It is for this reason that it is said that the polytenic chromosomes are found forming somatic pairs in a permanent prophase. In the female of *Drosophila*, for example, the polytenic chromosomes are found arranged in the following way. Chromosome pair Number I is composed of a straight cord. II and III, as pairs of chromosomes with two arms so placed that they both present a median inversion. pair IV is very small. Thus, there are found six elements in all (Fig. 73, 1 and 2). If they were separated one could count twelve elements: two chromosomes I, two chromosomes II with two arms, two chromosomes III with two arms, and two chromosomes IV of small size. In total eight chromosomes with twelve arms.

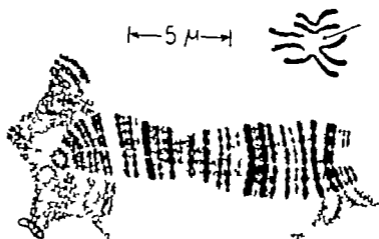


Fig. 72. The fourth polytenic chromosome of *Drosophila melanogaster* adhering to the chromocenter which is at the left. Above, at the right, the somatic chromosomes of the same fly as they appear in mitosis. The difference in size between the giant chromosome IV and the somatic chromosome IV indicated by the arrow and drawn to the same scale, is shown. (After Bridges, 1935)

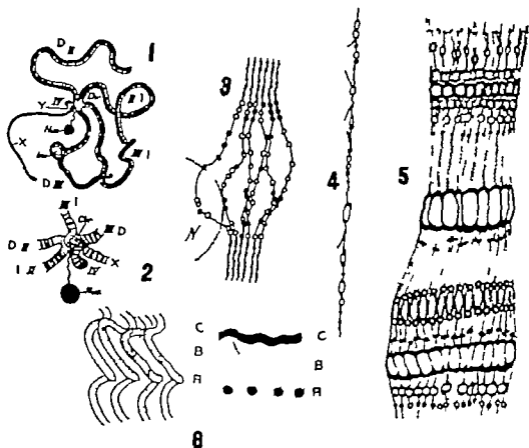


Fig. 73 Structure of the polytenic chromosomes. 1 General schematic aspect of the chromosomes of the salivary gland of a male of *Drosophila melanogaster* after they have been spread out by crushing the nucleus. The paternal chromosome (in white) and the maternal one (in black) are paired. Nuc, nucleus; Chr chromocenter X and Y indicate respectively the sex chromosomes; D II and II I right and left arms of chromosome II; D III and III I right and left arms of the third chromosome IV the fourth chromosome, Inv an inversion in the right arm

The *structure* of the polytenic chromosomes has much cytogenetic interest. Along the length of the chromosome there are a series of dark *bands* alternating with other clear zones called *interbands* or *internodes* which give to it the aspect of a striated muscle fiber (Figs. 71 and 72). The dark bands are stained intensely by substances which stain chromatin, and also by Feulgen's reaction. Furthermore, they absorb ultraviolet light very intensely (Fig. 71 *b*). These bands may be considered as discs, since they occupy the whole diameter of the chromosome. They are of varying size (broad or narrow) and result from the accumulation of chromomeres. The larger bands have a more complicated structure because the chromomeres are vesicular. This type of chromomere is called heterochromomere because it is usually found in bands of the heterochromatic regions. The *interbands* are of fibrillar aspect, do not stain with basic stains, are Feulgen negative, and absorb very little ultraviolet light. Furthermore, they present a greater elasticity than the regions of the bands. The constancy in situation and distribution of the discs or bands in the two homologous (paired) chromosomes is notable. The same chromosomes in any individual of the species in question possess an equal number of bands, with an identical distribution and localization. Thus it is easy to construct, from a giant chromosome, topographic maps of the bands and interbands, rigorously parallel to the genetic map (Chapter IX) and to verify any disarrangement or alteration in the order of their linear structure (Fig. 108). In the four chromosomes of *Drosophila* over 6000 bands are found. Detailed maps of each one of the chromosomes of this and other species have been drawn in which the genetic characteristics of each band and of the intermediate regions are meticulously recorded.

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of the third chromosome. 2 The chromocenter (*chr*) formed by the union of the heterochromatic parts of all the chromosomes in a female of *D. melanogaster*. The other symbols are the same as for 1. 3 A heterochromatic region of chromosome X of *Drosophila pseudoobscura*, showing its relations with the nucleolus indicated by  $\Delta$  and the filamentous (chromonemic) constitution of the chromosome. 4 Detail of a component chromonema of the polytenic chromosome in which the different chromomeres are seen. 5 Schematic structure of the chromosome of *Simulium* sp. *gatum*, showing the organization of the chromonemata, chromomeres and vesicles, which together give the appearance of the bands. The segment drawn corresponds to an euchromatic zone. 6 Diagram to illustrate the interpretation of the helical chromonema with the false chromomeres produced by the turns of the spiral. There is seen a zone (B) with four chromonemata, comprised between two consecutive bands (1 to the left) and the aspect presented by the same region when it is observed in a different focusing plane. In A a granular aspect is seen which simulates chromomeres, and in C a continuous solid line (1 and 2 after White 1943; 3 after Bauer 1936; 4 after Painter and Griffen, 1933; 5 after Painter 1946; 6 after Rus and Crouse 1945).

In regard to the *origin and formation* of these chromosomes, it will be recalled that in the holometabolous insects, the growth of the larval theme is generally carried out by increase in volume without any cell division. After the last mitotic division in the embryo the cells of the future salivary gland enter upon a long period of repose. Later there occurs a general growth of the cell. At the end of the period of growth, the individual chromosomes already are outlined in spiraled form. Then they become polarized and pairing begins. The homologues fuse, the thickness of the elements increases and the structure of the bands appears with great clarity. The peculiar constitution of these chromosomes is due to their formation from a number of fibers (four at the origin for each chromosome) which multiply many times, remaining together like the threads of a rope (Fig. 73, 3 4 and 5). Each fiber is very delicate and difficult to perceive and may be considered as a chromonema.

Micrurgical studies have permitted isolation of longitudinal fibrillae which show granules corresponding to the bands. Fraying of the chromosome into longitudinal fibrillae has also been accomplished by expelling a chromosome from a micropipette. A delicate elastic membrane around the chromosome was also demonstrated by microinjection techniques (D'Angelo).

The growth of these chromosomes is accomplished by the lateral splitting of the fibers. The same thing occurs with the bands or discs, which initially are four chromomeres and then proceed to increase in number and complexity until they form a large number of units of the same type (Fig. 73, 3 and 5). About nine reduplications are produced which lead to some 1000 fibers. The length of the chromonema is more or less that of a mitotic chromosome during the prophase, but in the giant chromosome, the chromonema is found drawn out, without spiralization. The discs or bands are chromomeres which, by uniting together, acquire the massive aspect which we already have described. There is no certainty that each one of the chromomeres represents a gene, since the size of the latter is probably even less than that of the smallest of the chromomeres of the polytenic chromonema. It is believed, nevertheless, that the genetically active part is found located in the region corresponding to the chromomeres, that is to say the bands.

In some cases, as in *Drosophila* (Fig. 73, 1 and 2) all of the polytenic chromosomes are found joined together at one place, called the chromocenter to which the nucleolus is also attached. This chromocenter seems to have been formed by fusion of the regions of the centromere of each one of the chromosomes. It appears to be an inert region composed of heterochromatin. On the other hand, Chromosomes or Sclera do not possess a chromocenter and the homologous chromosomes are more separated. Various authors have wished to associate the polytenic origin of the giant chromosomes with the fact that in the dipterous insects the phenomenon of endopoly ploidy is very common. This is nothing other than the multiplication, without mitosis, of the chromosome filaments. (See White 1945)

Giant chromosomes are also found in eggs of aquatic vertebrates. Chromosomes isolated from the germinal vesicle of the salamander egg measure over 0.8 mm. and may be stretched to as much as 1.9 mm. These chromosomes are not polytenic. They consist of only two chromatids to which double chromomeres are attached (Duryee).

## CHEMICAL COMPOSITION AND SUBMICROSCOPIC STRUCTURE OF THE CHROMOSOMES

(The chemical composition and submicroscopic structure of the chromosomes were outlined in Chapters IV and VII)

### Heteropyknosis and Heterochromatin

Closely related to the chemical dynamics of the chromosomes and, therefore, to metabolism of nucleic acid, is the phenomenon called *heteropyknosis* (Gr. *Heteros*, different, *Pyknos* dense)

It is common to find that certain chromosomes or parts of chromosomes during the interphase and prophase remain condensed and compact so that they stain intensely while other chromosomes or parts may appear more or less unstained. Such a tendency to condense is very general among the sex chromosomes (Figs. 83 and 109). This property characterized by differences in density of staining in various parts of the same chromosome was designated *heteropyknosis*. Areas staining more densely are spoken of as *positively* heteropyknotic, whereas the less dense portions are designated as *negatively* heteropyknotic. These variations in staining are believed to reflect greater or lesser concentrations of nucleic acid.



Fig. 74 Negative heteropyknosis of a chromosome (indicated by the arrow) during the meiotic metaphase I in the common toad, *Bufo arenarum*. (After Saez, Rojas and De Robertis, 1936.)

In the spermatogenesis of an orthopteran such as the locust *Schistocerca gregaria* both types of heteropyknosis appear clearly. In the weasel the same process also can be studied (Saez, 1930-1931). The phenomena of heteropyknosis may also be observed in other chromosomes besides the sex chromosomes. Heteropyknosis may be localized in certain segments or in the extremities, or intercalated in other zones of the chromosome or it may affect the whole or almost the whole chromosome. In the toad, *Bufo arenarum* Hensel there is found a chromosome with a negative heteropyknosis during the metaphase of the first meiotic division (Fig. 74). This element is not a sex chromosome but an ordinary chromosome (autosome) of the complex (Saez, Rojas and De Robertis, 1936).

In 1928 Heitz introduced the term *heterochromatin* to describe a particular type of chromatin which absorbs less stain than that generally found in chromosomes (*euchromatin*). Later he attempted to study the nature of the mechanism of the reaction of the heterochromatin and to assign a genetic basis to it (Heitz,

1935), outlining the hypothesis (which later fell into disfavor) that the heterochromatin is made of a genetically inert substance. Heterochromatin can be distinguished in prophase and in metaphase, although with a different heteropyknotic reaction. In the prophase the heterochromatic segments may contain compact darkly staining bodies looking like small nucleoli. At first they were called *prochromosomes*, later it was postulated that they might be condensed parts of certain chromosomes and they were given the name of *chromocenters* or *euchromocenters* (Fig. 75 a)



Fig. 75 Effects produced by the action of cold on the chromosomes. *a*, Nucleus of the embryo sac of *Fritillaria pudica* in the prophase, showing the three heterochromatic bodies stained black (in positive heteropyknosis) with abundant nucleic acid. The nucleolus, which is seen as a large sphere, has not been colored by the Feulgen's stain and its color is due to the osmic acid with which the cell has been fixed. *b* Metaphase chromosomes of *Fritillaria pudica*, showing the clear zones produced by the action of a temperature of 0 C. These zones correspond to the heterochromatin (in this case a negative heteropyknosis). The dark part of the chromosome corresponds to the euchromatin. (After Darlington and La Cour 1941.)

The differential staining reaction or heteropyknosis of the heterochromatin has been recently studied in relation to the cycle of the union of nucleic acid to the protein chain of the chromosome. Darlington and La Cour have found that by bringing about cell division at temperatures between 0 and 3 C it is possible to produce, in the metaphase chromosomes, the formation of clear unstained segments which under normal conditions appear optically homogeneous and undifferentiated (Fig. 76 b). These regions are considered to be heterochromatic. Darlington has designated as *allocycli* this specific differential reactivity of the heterochromatin. With this method it is possible to investigate heterochromatic zones in the chromosomes of an organism and to learn more about the metabolism of nucleic acid. It has been shown that the clear segments, having discharged their nucleic acid, show deficiencies during cell division, which supports the belief that nucleic acid plays an important role in the production of the genes.

The constancy of distribution of such clear segments makes them valuable for the characterization of each chromosome of the group. Heterochromatic segments produced by low temperature appear in the prophase to be condensed, strongly basophilic and Feulgen positive thus resembling true chromocenters (Fig. 75, a)

The heterochromatin is not now considered to be an inert zone of the chromosome since its active participation in nucleic acid metabolism suggests that perhaps it may play an important role in the control of development. Caspersen has postulated that the heterochromatic regions are composed by genes or similar elements. This would explain certain alterations such as duplication or deficiencies which are less harmful to the organism than those which occur in the euchromatic segments. Darlington has expressed the idea that the difference existing between the activity of the two regions rests on the high and the low specificity of the euchromatin and the heterochromatin respectively. The behavior of the supernumerary chromosomes favors this view. Although considered genetically inert they may not necessarily be physiologically inert. The Y chromosome of *Drosophila* presents an extensive heterochromatic region and carries few genes. When this chromosome is found as an extra element in the female, giving it the constitution XXY rather than XX, the quantity of the cytoplasmic nucleotides is increased in the egg of this insect. This indicates then that the Y chromosome may carry out an important function in the regulation of the metabolism of nucleic acid of the cell (Caspersen and Schultz, 1938).

The question of the origin of the heterochromatic segments is still under dispute. One presumption is that they are formed by the repeated reduplications of euchromatic regions (chromomeres or genes). This interpretation is supported by the work of Vather who, on genetic grounds, accounts for the homogeneous structure of the heterochromatin as due to the formation of *polygenes*, which are merely the repetition or replicas of a gene. For a small part of the chromosome, such as a gene or chromomere, numerous possibilities exist during the first linear duplication for new heterochromatic blocks to be propagated and localized. Furthermore, those already formed may be separated and distributed, becoming intercalated between the euchromatic segments (Pontecorvo, 1944; Saez, 1945).

## CELL DIVISION

The growth and development of every living organism is conditioned by the growth and multiplication of cells. In the unicellular organisms cell division implies a true reproduction and, by this process, two or more new individuals arise from the original. On the other hand, the multicellular organisms come from a single primordial cell, the zygote, and it is the repeated multiplication of this cell and of its descendants which determines the development and growth of the individual.

The size of any organism is determined by the number of cellular elements which compose it and not by the volume of individual cells. The nature of the processes which determine the size and the form of the organism is, for the most part, unknown except that it is genetic in nature. Each class of cells shows a general uniformity of volume which may differ markedly from the volumes of cells of a different nature. In many instances cells appear to grow up to a limit which, having been attained, is followed by division into two daughter cells. The two cells thus formed may grow until they reach their limit of volume and then again divide, giving rise to a second generation of four cells whose total volume will eventually become four times that of the original cell, and so forth, successively. In other words, the

the mother star or the monaster) The achromatic spindle is completely formed.

### Anaphase

The diverging movement of the longitudinal halves of the chromosomes begins these are the *chromatids* which separate, directing themselves toward their respective poles, as daughter chromosomes

### Telophase

The daughter chromosomes reach the poles of the spindle and here they come together maintaining a parallel arrangement. The nuclear membranes are reformed and the nucleoli reappear. The stages in nuclear reconstruction during this phase occur in inverse order to those which characterize the prophase. There commences, at the same time, the symmetrical segmentation and separation of the cell body or cytoplasm of the cell, a process called *cytokinesis* *cytodieresis* (Gr *Dieresis* division) or *plasmodieresis* and two new cells are formed, the nuclei of which begin the interphase, the process then repeating itself. The nucleoli reappear at the nucleolar organizer of the nucleolar chromosomes

### Analysis of the Mitotic Process

During the interphasic stage, the chromosomes are difficult to observe in the majority of the cells, a fact which does not signify that they are absent or that they have lost their characteristic organization. The perceptibility of the chromosomes is greater or less according to the physiological states through which the nucleus passes during division, and the fact that their visibility is at a minimum during the interphase led to an early belief that these elements were formed anew each time that the cell entered upon a mitosis. Later there appeared clear demonstrations of the presence of chromosomes during all the stages of cellular life. In the interphase they have been demonstrated particularly well in the insects, especially in the Orthoptera and Hemiptera. The most notable example is that of the sex chromosomes, whose heteropyknosis makes them appear during this stage as dense bodies in the midst of the other chromosomes, which remain scarcely visible. Another case indicating the permanency of the chromosomes through the entire nuclear cycle is the finding in the interphasic nuclei of some plants and animals of the *prochromosomes* or *chromocenters* bodies which correspond to the heterochromatic regions of the chromosomes

In the interphasic nuclei of *Arrhenatherum*, *Festuca* and some other genera of the Gramineae it has been possible to follow in vivo the course of the chromosomes, which appear as very delicate filaments. By an analysis of the process of spurlization which each chromosome carries out during the mitotic cycle and the meiotic cycle, it has also been established that they do not disappear at any time and that the different aspects which they present are related to their internal mechanics.

The *prophase* is marked by changes in the physicochemical state of the cytoplasm. The cell tends to adopt a spherical form, acquires a turgidity and increases its refractivity. These changes are accompanied by a rise of surface tension and viscosity. At the same time the viscosity of the karyoplasm often seems to diminish. These physicochemical modifications would facilitate the later changes and movements of the chromosomes. The pro-phasic chromosomes appear as delicate longitudinally spiral filaments, doubled and extended or making turns in the nuclear sphere, according to their lesser or greater length (Fig 76 a, b c). The whole group of chromosomal filaments, particularly when they are numerous and long presents the aspect of a ball of yarn carelessly rolled together inside the nuclear cavity. For a long time it was believed that this ball was composed of a continuous filament, the *spireme* of the classical authors, which, later, would undergo a transverse segmentation, thus producing a certain number of segments of varying length, the chromosomes. This erroneous concept of the continuous spireme persists even now in numerous works, particularly in those designed for teaching, in spite of the fact that there exists no objective basis for it. The name, as well as the interpretation, of this stage has been definitely abandoned. There are numerous cytological and genetic proofs which make insupportable the hypothesis of a continuous filament. Each chromosome possesses a special differential organization and maintains through the entire nuclear cycle its autonomy and its specific functioning.

It has been explained that each prophasic chromosome is composed of two spiral filaments called *chromatids* which are found closely associated throughout their entire extent without actually fusing (Fig 76 c d e). As the prophase progresses, the chromatids undergo shortening. This shortening might be due to a rolling up of their internal spirals, to desolvation of the protein substance of the chromosome and to an increase in the pH of the karyoplasm. As they shorten they increase in volume, perhaps in part because of an accumulation of nucleic acid by the transference of nucleotides from the cytoplasm to the chromosomes. The appearance of the chromosomes in the prophase stage depends in part upon the duration of the preceding interphase.

When this has been short and the number of chromosomes of the species is small, it is found that the positions occupied by each

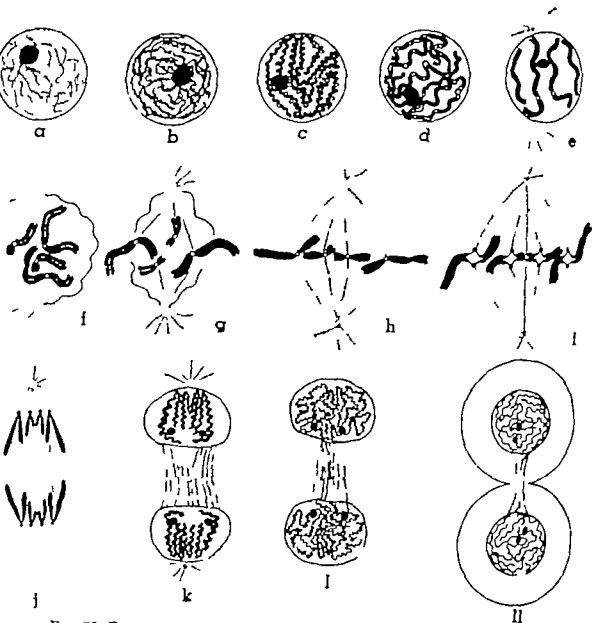


Fig. 76 Diagram of the different stages of mitosis *a*, interphase; *b c, d, e*, prophase in which there is observed a progressive contraction and condensation of the chromosomes (each one composed of two chromatids); *f g* prometaphase, the spindle is beginning to form and the nuclear membrane is disappearing; *h, i*, metaphase; *j* anaphase; *k, l, ll*, telophase. The centromere is indicated by a clear circle in each one of the chromosomes.

prophasic chromosome is the same as its place in the telophase of the preceding division.

Upon careful observation one can see the centromere in a constant position in each chromosome as a small clear circular zone (Fig 76) This region appears to play a fundamental role in

the movement of the chromosomes and maintains a close dynamic relation to the cell centers or poles. With the increase in volume of the chromosomes, the centromeric region becomes more accentuated and appears in the metaphase as a constriction, the *centric* or *primary constriction*.

In early prophase when the chromosomes are long, their constituent chromatids coil together as a general rule (Fig 76 e). During the entire prophase, the chromosomes are separately distributed in the nuclear cavity. This separation increases as the prophase progresses and the chromosomes tend to approach the internal edge of the nuclear membrane, leaving the central space of the nucleus clear. The centrifugal movement of the chromosomes indicates that the disintegration of the nuclear membrane is approaching and with it the end of the prophase. The shortening of the chromosomes reaches almost its maximum and the length of some of them may shrink to one twenty fifth of the length seen in early prophase. At the end of the prophase each chromosome appears to be composed of two cylindrical longitudinal elements called the longitudinal halves, parallel to each other and in close proximity.

The formation of the spindle shows a number of variations. In one type of spindle formation, known as the central spindle, the achromatic figure commences to make its appearance with the activity of the *centriole* which lies at one side of the nuclear membrane. This body divides into two daughter centrioles which begin to separate while the prophasic phenomena are occurring within the nucleus (Fig 46). After this division, there often appears above each one of them a system of radiations called the *asters* or *astral rays*, and, arising between the two asters, there is a bundle of delicate filaments, the *spindle*. The centrioles continue their migration, along with the asters, describing a semicircular path toward the poles, until they become situated in antipodal positions (Fig 76 c f). There exists another type of spindle formation, *metaphasic spindle* in which the centrioles are already polarized before the beginning of division, the spindle being formed when the chromosomes begin their metaphasic arrangement (Fig 76 g). The mitoses in which the achromatic figure and spindle is formed by centers (centrioles and asters) receive the name of *astral* or *amphiastral* mitoses, and are common in animal cells and in some lower plants. Mitoses which lack the centers are called *anastral*, and are characteristic of the great majority of plants (Fig 77 3). There is no unanimous agreement concerning the origin of the achromatic apparatus and the problem is under discussion as to whether nuclear materials intervene

in it or whether it is only formed at the expense of cytoplasmic materials. Whatever the conditions may be which preside over the formation of the spindle after fixation, its aspect is fibrillar showing delicate threads which pass from one to the other pole or from the poles to the chromosomes. These fibers are not seen *in vivo* and for this reason, have been subjected to discussion. However, submicroscopic methods have revealed the presence of an underlying fibrillar organization. (See Chapter IV) By the use of centrifugal force applied to dividing cells it has been demonstrated that the achromatic figure is a rigid system which is displaced through the cytoplasm without losing its integrity (see Saez, 1941)

The *prometaphase* generally begins with the disintegration of the nuclear membrane, although there are cases of intranuclear mitoses among the Protozoa and in some other groups in which mitosis is carried to completion without the disappearance of the membrane. When the nuclear membrane has disintegrated, a more fluid zone is noted in the center of the cell in which the chromosomes are freely movable and begin to be displaced in apparent disorder toward the equator. This mechanism of equatorial arrangement was called *metakinesis* (Wassermann)

*Metaphase* begins when the translation of the chromosomes ceases and they reach the plane of the equator where they may arrange themselves in a radial manner at the periphery of the spindle as if they were mutually repelling each other (Fig 76, *h*). This arrangement is characteristic of animals (Fig 80, 1, 5) though in plants the chromosomes are irregularly arranged and occupy the entire surface of the equatorial plane of the spindle (Fig 80 3 7). The distinction between these two types of arrangement is not always clear. In both types of arrangement, if small chromosomes occur in the group, they are commonly situated toward the interior while the larger ones are customarily found peripherally (Fig 80, 2 4 6). The part of the chromosome directed toward the axis of the spindle is called the *proximal part*, while the opposite part is called the *distal part*.

The array of chromosomes on the spindle is called the *equatorial plate*. In this stage, the chromosomes are connected to the fibers of the spindle by means of their centromeres. Those fibers of the spindle which connect to chromosomes are called the *chromosomal fibers* (Fig 77 *c*) while those which extend without interruption from one pole of the spindle to the other are called the *continuous fibers* (Fig 77 *e*). When the chromosomes are observed in polar view one can determine with relative facility their number, form and dimensions. These properties are

characteristic for each species and constitute its *karyotype* (Fig 80)

The equilibrium of forces which characterizes the metaphasic state is broken by the division of the centromere by which the chromatids have been united up to this time. The division of the

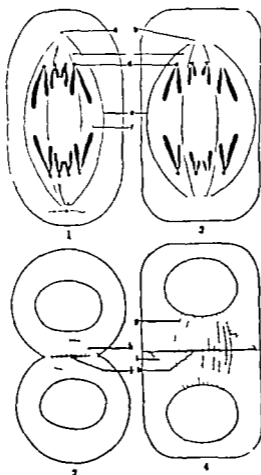


Fig. 77 Diagram of the constitution of the spindle during the anaphase and telophase in an animal cell (1 and 2) and a plant cell (3 and 4) *a*, centriole, *b* polar center, *c*, tractor or chromosome fiber, *d*, centromere or kinetochore, *e* continuous fiber of the spindle; *f* fiber or interzonal connections; *g* spindle of cytokinesis; *h*, remains of the spindle; *i*, phragmoplast, *j* intermediate bodies, *k*, cell plate 1 anaphase, 2 telophase with cytokinesis by constriction, 3 anaphase, 4 telophase with cytokinesis by formation of the cell plate.

centromeres is carried out simultaneously in all of the chromosomes (Fig 76 *i*). The daughter centromeres move apart and, in their turn, the chromatids separate, beginning their migration toward the poles (Fig 76 *j*).

This process characterizes the beginning of the *anaphase*. The diverging movement of the chromatids seems to be autonomous, produced apparently by an accentuated repulsion between the daughter centromeres. From the time the chromatids

separate they are called *daughter chromosomes*. They shorten longitudinally and the longest chromosomes still remain in contact by their distal extremities for a time after the short chromosomes become separated. Even this contact ceases when the groups of daughter chromosomes approach closer to their respective poles.

During the latter half of the anaphase a change occurs in the appearance of the achromatic spindle. In the zone between the two groups of chromosomes the spindle fibers appear stretched, and a group of fibers called *interzonal fibers* appears (Figs. 76, j and 77, f). This apparent expansion of the middle part of the spindle, which was called *Stemmkörper* by Belar can be translated as the *pushing body* or *pushing spindle*. It is interpreted as a kind of solidified gel which drives the daughter chromosomes toward the poles at the end of the anaphase (Fig. 76, j k). However other mechanisms for the separation of the daughter chromosomes have been postulated.

The anaphasic changes transform the centrosomic spindle between two points into a centrosomic spindle between two plates (Darlington).

The end of polar migration of the two daughter groups marks the beginning of *telophase*. In this stage, favorable preparations may show the spiralized structure of the chromosomes with their *chromonemata* (Figs. 76, k, l and 79 7 8). A little later the processes of nuclear reconstruction occur. These appear to be prophase processes in reverse. The chromosomes lose their state of condensation, the spirals of their chromonemata becoming unwound (Figs. 76 l, ll and 79 8). An imbibition from the surrounding karyoplasm occurs, and with it a dissociation of the envelope or *matrix* of the chromosome, which generally becomes invisible.\*

The membranes of the daughter nuclei are reconstructed while the telophasic chromosomes are transformed. During the final stages, the nucleoli reappear at the *nucleolar organizers* or *SAT zones* (Fig. 76 k, l, ll).

Next there occurs the process of segmentation and separation of the cytoplasm, or *cytokinesis*. In animal cells the cytoplasm constricts in the equatorial region and this constriction is ac-

In many cases, nevertheless, the chromosomes remain visible during the whole interphasic stage, retaining their relative positions within the nucleus up to the commencement of the next mitosis. In the spermatogonial cells of some orthopterous insects, as also in some Protozoa, the chromosomes form separate vesicles, which persist during the interphase. Belar (1929) could follow the mitotic process in living cells of the staminal hairs of *Tradescantia*, proving that there exists a correspondence between the arrangement of the chromosomes in the telophase and in the following prophase.

centuated and deepened until the cell takes the form of an "8" leaving a delicate bridge which, on its disappearance, sets at liberty the two newly formed cells (Figs 76, 11 and 77 2) In plant cells, which possess rigid membranes, the fibers of the spindle vanish progressively from the central to the marginal zone, and the intermediate body called the *cell plate* persists between the two daughter cells, forming the cell membrane At this moment the spindle presents an aspect similar to that of a barrel and is called *phragmoplast* (Fig 77 4)

Other cytoplasmic changes also occur in telophase. The high viscosity characteristic of metaphase and anaphase decreases during the telophase. The centrosomes cease their activity and the asters become less conspicuous During cytokinesis the distribution of the formed components of the cytoplasm takes place The division of the chondriome is called chondriokinesis (Meves) and that of the Golgi apparatus, dictyokinesis (Perroncito) or golgi okinesis (Sosa) It has not been demonstrated that the partition of these organoids is absolutely equal, as in the case of the chromosomes, although it is possible that it may be

### Duration of the Mitotic Cycle

The duration of the complete cycle of mitosis depends upon the physiological conditions of the organism, as well as upon certain external factors such as temperature, and is also variable in the different cells of the same species. (See Table VII)

TABLE VII  
DURATION OF THE MITOTIC CYCLE (Time in Minutes)  
(From Schrader 1944)

	Segmentation in <i>Drosophila</i> (Fixed C II) Rabinowitz, 1931	Mesenchyma of Chicken (Living Cells) Lewis & Lewis, 1917	Plant, <i>Arrhen- therum</i> (Living Cells) Martens, 1937
Interphase	29	30-120	
Prophase	36	30-60	36-45
Metaphase	5	2-10	7-10
Anaphase	12	2-5	15-20
Telophase	9	5-12	20-33
Total	91	67-203	78-110

### Cycle of the Nucleolus in Cell Division

Up until recently the role played by the nucleolus during mitosis constituted a difficult problem Its disappearance at cer

tain stages of the process of division (metaphase and anaphase) and its presence in the interphasic and prophasic stages has not been understood until recently. In describing the structure of the chromosomes, it was said that there exists at least one pair of chromosomes, called the nucleolar chromosomes, each one of which produces a nucleolus. These nucleoli originate at the end of the anaphase or in the telophase at the nucleolar organizer or SAT zone. At the beginning of its formation, the nucleolus appears as a small granule situated in this particular zone of the chromosome (Fig. 78, 1). This granule does not stain by the

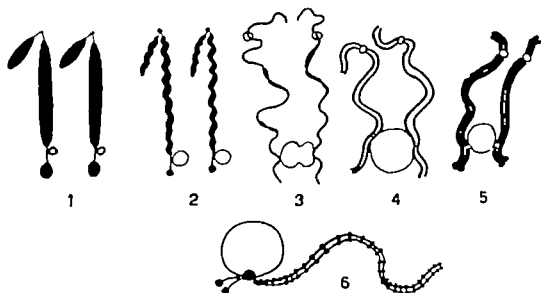


Fig. 78. Cycle of the nucleolus during cell division. 1 anaphase of a pair of nucleolar chromosomes in which the satellites and the nucleolar zone appear; 2 telophase; 3 early prophase with the fused nucleoli; 4 middle prophase; 5 advanced prophase; 6 nucleolar chromosomes in the pachytene stage during the meiotic prophase showing the nucleolar organizer, the nucleolar body and the filament of the satellites with the satellites.

Feulgen method while the rest of the chromosome does. Gradually the volume of each nucleolus increases (Fig. 78, 2) and later the corresponding nucleoli generally fuse into a single mass (Fig. 78, 3, 4, 5). They remain united in this manner until the beginning of the next prophase, in which stage the nucleolus is seen in its typical globular form (Fig. 78, 4 and 5). The chromosomes which gave it origin remain associated with it by the same regions which contributed to its formation (Fig. 78, 4 and 5).

At the moment of the disintegration of the nuclear membrane, the nucleolus is liberated from the nucleolar chromosomes, disappearing immediately afterwards. A large part of its constituent material is apparently employed in the reconstruction of the sheath of the chromosome, while the remaining part seems

to diffuse into the cytoplasm. The nucleolus, as the sheath of the chromosome, contains basic proteins of the histone type. Very probably the nucleolar zone of the chromosomes is important in

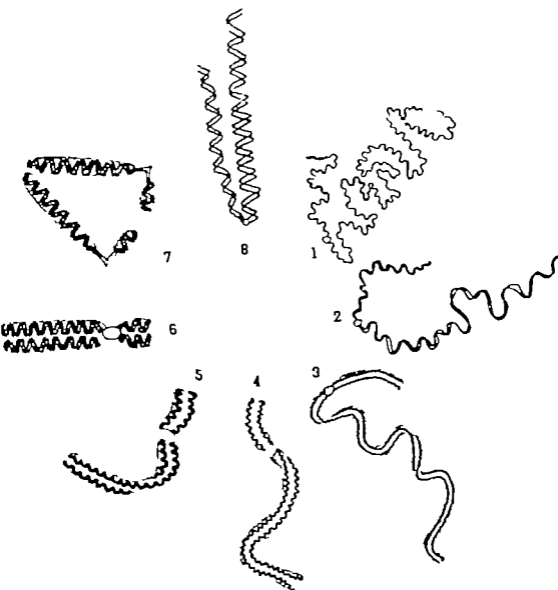


Fig. 79 Diagram of the cycle of spiralization of the chromonema during mitosis 1 interphase with the relic spiral and the superspirals 2 3 4 prophase showing the relic spiral and the formation of the matrix corresponding to each chromonema 5 prometaphase with the chromonema of each chromatid duplicated, 6 metaphase showing each chromatid with two chromonemata in which appear both the major and the minor spiral; 7 anaphase 8 telophase. The centromere has been indicated by a clear circle.

the organization of the proteins and of the nucleic (ribonucleic) acid which the nucleolus contains. The nucleolus may be a site of provision of proteins and of nucleic acid for the matrix (See Chapter VII)

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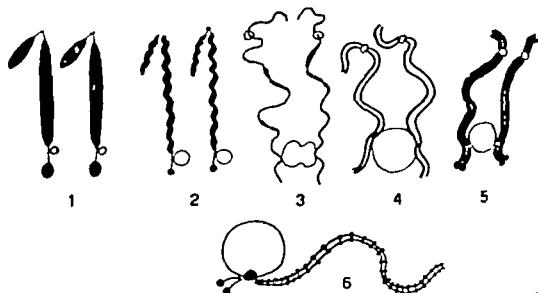


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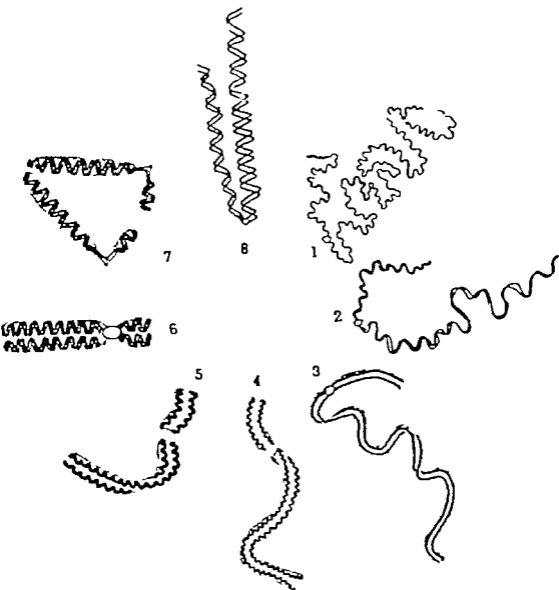


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## The Cycle of Spiralization of the Chromonema

The cycle of spiralization of the chromonema is carried out regularly during mitosis. In the interphase the chromonemata show minimal spiralization. At this time the delicacy of the filament is such that it may be broken by the action of fixatives. In this case the nucleus presents the classical picture which led to the interpretation of nuclear structure as a reticulum. Due to the lengthening of the chromonema and the restriction of the nuclear space, the filament takes the form of a zigzag with numerous tortuosities constituting the *superspirals* (Fig. 79, 1).

During the prophase each chromosome is formed of two chromonemata wound into large spirals (in addition to the superspirals which progressively disappear) which, as the stage progresses, diminish in number and in amplitude until they disappear. These spirals are those which remain from the previous division and for which the designation of *relic spirals* is used (Fig. 79 2 3). Meanwhile, during the middle of the prophase and before the relic spirals become completely unwound, there appears a new spiral, the *major spiral* which is supposed to be derived from a *molecular spiral*. This new spiral develops gradually by an increase of the diameter of the turns while their number diminishes (Table VIII). Simultaneously the length of the chromatids likewise is diminishing (Fig. 79 2 3 4 5). The process continues in this way until the maximum winding up and contraction in the metaphase (Fig. 79 6).

In summary it may be said that the contraction of the filaments which make up the chromatids is accompanied by two contrasting processes, (1) the loosening of the relic spirals which come from the preceding anaphase; and (2) the development of a new spiral in each chromatid. This latter spiral, once it has been completely formed, will constitute the *major spiral*. At the beginning of the metaphase the *minor spiral* also appears and, at the end of the anaphase, the *major spiral* gradually loosens. This process continues during the telophase and interphase. The rest of this spiral forms the relic spiral.

The constitution of each chromatid can be traced from the first stages of the prophase. It will be remembered that each of the two chromatids was constituted by a chromonema. They persist in this condition until the prometaphase. During the prometaphase the chromonema of each chromatid is duplicated so that, from this

TABLE VIII

LENGTH IN MICRONS AND NUMBER OF TURNS OF THE CHROMATIDS OF *Trillium grandiflorum* DURING THE MITOSIS OF POLLEN GRAINS (MICROSPORES)  
(From data taken from SPARTOW 1942.)

Condition	Length of the Chromatids	Number of Turns
Relic spiral of 60 turns	450-600	450-600
Prophase	516	551
Middle prophase	202	212
Middle prophase	203	278
Final prophase	173	131
Final prophase	184	170
Final prophase	142	187
Metaphase	77	190
Anaphase (15 cells, average)	95.0 $\pm$ 2.9	150 $\pm$ 3.3

We have said above that the number of filaments or chromonemata which make up a chromosome is still a matter of discussion. In the figures which illustrate the process of spiralization, we have considered that each chromosome is composed during the interphase of two chromonemata.

time on, each chromatid contains two chromonemata. Later a metaphasic chromosome will be formed by two chromatids very close together each with two chromonemata, in all, four chromonemata (Fig. 79.6). When the anaphasic separation is produced, the chromatids separate, each one bearing two chromonemata (Fig. 79.7) and pass to their respective poles to enter into the telophase, whereupon a chromosome (the name now given to the chromatid) forms from the two daughter chromonemata.

It is interesting to note that the duplication observed in the prometaphase takes place before the separation of the chromatids in the anaphase (Fig. 79.5). It is presumed from this that the duplication has been produced submicroscopically much earlier but that it can be seen for the first time in the prometaphase.

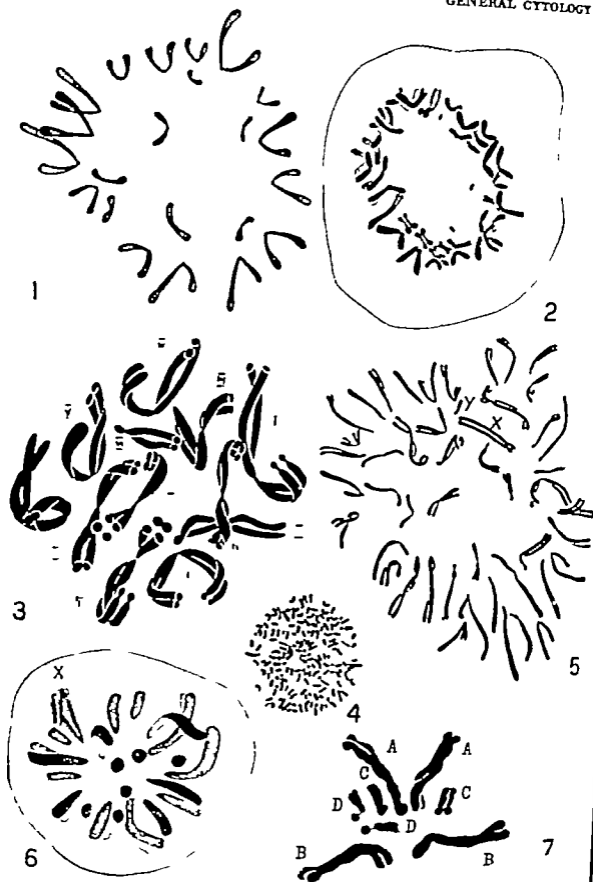
During the anaphase, telophase, interphase and prophase, the chromosomes are double (two chromonemata in each one). In the prometaphase and metaphase they are quadruple since each one has four chromonemata.

## CHARACTERISTICS OF THE CHROMOSOMES

The most important characteristics identifying individual chromosomes in mitosis are their number, relative dimensions, structure, behavior and internal organization. There are other characteristics such as the linear contraction, the degree of spiralization and the volume, which are subject to physiological variations. Furthermore, the chromosomes are controlled by genetic conditions in the individual. This is what is called the genotypic control.\*

The number of the chromosomes is one of the best known constants. Generally organisms are constituted by a *diploid* somatic complex of chromosomes, arising by the union of the two gametes. Each one of these gametes contributes one set of chromosomes called *haploid*. Thus, in the human species (Fig. 80.5) the somatic or diploid number of chromosomes is 48. This number is formed by two haploid sets of parental origin, each with 24 chromosomes. Each haploid set of chromosomes is designated by  $n$  and the diploid by  $2n$ . Some organisms have a somatic number of 24 chromosomes in some individuals, and 36 or 48 in others. In these cases, the haploid sets also vary, possessing respectively 12, 18 and 24 chromosomes each. Under these circumstances, the lowest haploid number (in this case 12) is called the *genome*. Each one of these sets or genomes is conventionally designated by  $x$ . In the example given, if  $x$  is 12, the individuals with 24 somatic chromosomes have two genomes of 12 chromosomes each ( $2x = 24$ ); the individuals with 36 somatic chromosomes possess three genomes ( $3x = 36$ ) and those with 48 chromosomes are formed by four genomes ( $4x = 48$ ). Supposing that the individuals with 48 chromosomes originated by the fusion of two gametes with  $n = 2x$  each, each individual will have the following chromosomal formula:  $2n = 4x = 48$ . It is customary also

*Genotype*: Fundamental hereditary constitution of an organism.



See Facing Page 191 for Caption to this Cut

to represent this case thus  $2n = 48$  or  $4x = 48$ , although the former annotation is more correct, since it indicates the number of genomes which have given rise to the organism in question

There are cases of organisms which have originated from a nucleus with the haploid number of chromosomes. Such organisms, called haploid, may be brought about by parthenogenesis or by apogamy without the intervention of gametes, but never by fertilization.

The number of chromosomes serves as an aid for the determination of phylogenetic relationships, such as the taxonomic position of plant and animal species

The organism which has the smallest number of chromosomes is the nematode *Acaris megalocephala* of the variety *univulvis*, with two somatic chromosomes and therefore  $n = 1$ . Among animals, the highest numbers are found in the Protozoa of the group *Agregata* which possess over 300. The moth *Philgala palaria* has 224 somatic chromosomes. The numbers most common among the plants are 12, 14, 16, 18 and 24 and among the animals, 12, 24 and 40 are the diploid numbers.

The form of the chromosomes also is a characteristic which is of aid for the identification of a particular complex. Some species have chromosomes which can be identified with ease (Fig. 80)

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Fig. 80 The characteristics of the somatic chromosomes of some plants and animals. All the figures correspond to metaphases in polar view. 1 the 22 chromosomes of the toad *Bufo arenarum* showing their morphology. In some chromosomes a black point corresponding to the centromere is seen; 2 the chromosomes of a reptile *Tupamabis teguixin* showing their 140 elements distributed as microchromosomes in the center and macrochromosomes at the periphery. 3 the complex of chromosomes composed of 12 elements of a member of the Ranunculaceae, *Nigella orientalis* in which some chromatids can be seen wound about each other thus forming the relational spiral. The Roman numerals indicate the pairs of homologous chromosomes. 4 the 208 chromosomes of the decapod crustacean *Paralithodes camischottii*, showing their rod-shaped and punctiform morphology; 5 the 48 chromosomes of the human species showing, in the upper part of the plate of chromosomes, the sex chromosomes X and Y. One may note the relational spiralization of some elements and the centric and secondary constrictions. 6 the 23 telocentric chromosomes of the locust *Chromocris miles*; the sex chromosome indicated by X is found in negative heteropyknosis. 7 the 8 chromosomes of the composite plant *Hypochaeris lucidus* showing a pair of satellites. The letters indicate the pairs of homologous chromosomes. 1 from Saez, Rojas and De Robertis (1936); 2 from M. Hey (1933); 3 from Lewitsky (1931); 4 from N'jama (1935); 5 from La Cour (1944); 6 from Saez (1930); 7 from Saez (1944).

Concerning the number of the chromosomes in plants G. Tischler has published lists in *Tabulae Biologicae*, Vol. 4 (1927); 7 (1931); 11 (1936) and Gaiser in *Bibliographia Genetica*, Vol. 6 (1926); 10 (1933) and *Genetica*, Vol. 8 (1926). Concerning the number of chromosomes in animals, H. Hey has published lists in the *Journal of Morphology*, Vol. 23 (1917); 34 (1920); Oguma and Nakino, in the *Journal of Genetics*, Vol. 26 (1931) and the *Journal of the Faculty of Sciences, Hokkaido Imperial University*, Vol. 5 (1937). Bräslau Hirsch, in *Tabulae Biologicae*, Vol. 4 (1927) and McClung, in *Tabulae Biologicae*, Vol. 28 (1940). See Darlington and Janaki Ammal (1945) for cultivated plants.

1, 3, 6 7) while in others it is practically impossible to distinguish the different chromosomes of a cell (Fig 80, 2, 4)

Butterflies generally have small dotlike chromosomes similar to one another. In some composite plants such as *Crepis* and *Hypochoeris*, in other groups such as the genera *Muscari* and *Vicia* and among certain insects, especially the *Diptera*, the configuration of the different chromosomes can be studied separately (Fig. 80, 7)

The form of the chromosomes may undergo alterations through mechanical, chemical, or radiational accidents, or during cell division, or by experimental means. Furthermore, spontaneous alterations may occur in nature, it being difficult to determine their origin and their difference from the accidental ones. The criterion employed for the morphological identification is based upon the position of the centromere, on the secondary constrictions, and on the existence and localization of satellites.

There are zoological groups which present a typical morphology such as the family of *Acrididae* (locusts) the chromosomes of which are always telocentric and rodlike (Fig 80, 6) or the amphibia (Fig 80 1) which have metacentric chromosomes, each in the form of a V. Among the plants the form of the chromosomes is more varied with characteristic satellites and constrictions (Fig 80 3, 7)

The best condition for determining the form of a chromosome is the metaphase or early anaphase generally in polar view (Fig 80), but in some cases in lateral view. When one is dealing with meiotic chromosomes it is necessary to observe their configuration from various angles in order to obtain a clear image of their morphology.

The *size of the chromosomes* is relatively constant and is important in the individualization of each one of the members of a complex. The relative dimensions of the chromosomes of a cell usually differ among themselves but at times all the chromosomes of a group may be of the same size. The length of a chromosome may vary from 0.20 up to 50  $\mu$ , the diameter varying between 0.20 and 2  $\mu$ . In the human species the approximate length of the chromosomes is 4 to 6  $\mu$ .

A diploid complex of chromosomes consists of a double series of elements or pairs which correspond exactly in form and size. In *Hypochoeris* for example (Fig 80, 7) there are eight somatic or diploid chromosomes divided into four pairs AA BB CCDD of chromosomes. Each member of each pair is similar to its mate and thus they are called *homologues*. In man there are 24 pairs of homologues in the toad and the weasel, 11 in the fly *Drosophila*, 4 in the onion, 8 in corn, 10 and in tobacco, 12

Each homologue is not invariably found in intimate contact with its mate, since the location of each chromosome at different periods of the mitotic cell cycle is entirely independent. A given chromosome may occupy any part of the nucleus. Dipterous insects are exceptional in that the homologous chromosomes commonly occur together in polytemic nuclei. Furthermore, in the common somatic cells of these insects, the homologous chromosomes are found close to each other in the prophase and metaphase, although they do not touch. This condition is designated *somatic pairing* (Fig 110)

The name *karyotype* is given to the group of characteristics including the number, the form, and the size and other characteristics which may be taken into consideration in identifying a particular chromosomal complex. Thus karyotype is characteristic of an individual, a race, species, genus, or larger grouping. The karyotype may be represented by a diagram called an *idiogram*.

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## Chapter IX

### CYTOGENETICS

*Cytogenetics* one of the most recent of the biological sciences, arose by the correlation of cytology with genetics. It represents a broad and promising field of study with great theoretical and applied importance. In the preceding chapter some of these problems have been studied, particularly those related to the chromosomes. In this chapter we shall consider the main aspects of cytogenetics in relation to heredity, sex determination, mutation, evolution and systematics.

#### MEIOSIS

In the preceding chapter it was explained that the chromosomes are regularly distributed by means of mitotic cell divisions, which generally maintain the constancy of their number through all the cells of the individual. Since the somatic cells of most organisms are derived by mitosis from a fertilized egg or zygote, they all possess a double (*diploid*) set of chromosomes. The gametes (ovum and spermatozoon), however, arise by a special type of cell division, called *meiosis*, in which the normal diploid set of chromosomes is reduced to a single (*haploid*) set. The meiotic process characterizes all plants and animals which reproduce sexually. It is in the course of gametogenesis, during the maturation of the sex cells, that meiosis takes place. In essence, meiosis consists of *two nuclear divisions which follow each other rapidly while the chromosomes divide only once*. The result of this process is the formation of four nuclei, each one of which has a simple or haploid number of chromosomes. These two divisions are called the *first meiotic division* and the *second meiotic division* or simply I and II (Fig 81 5 and 7). The purpose of meiosis is not only the numerical reduction of the chromosomes but also the attraction, the pairing, interchange and separation of the homologous paternal and maternal chromosomes.

The first meiotic division is characterized by a long prophase during which the chromosomes pair closely and effect an interchange of hereditary material between the two conjugating members (Fig 81 3). As a consequence of this conjugation (*synapsis*) the pairs of homologues appear as double or *bivalent* chromosomes. If for example, there were six chromosomes in the diploid

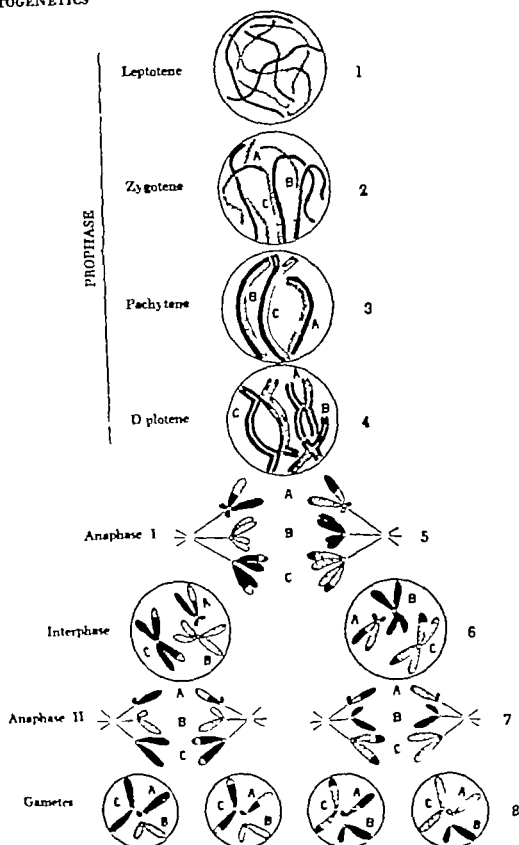


Fig. 81 General diagram of meiosis which illustrates the union, separation and distribution of the chromosomes.

set, A, B, C, A, B, C (Fig 81, 1) when the pairing is completed three bivalent chromosomes will be encountered, AA, BB CC (Fig 81, 3) Under these conditions, the prophase continues until there is a longitudinal cleavage of each one of the homologues but without a total separation of their halves or *chromatids*. At this time the nucleus shows only three bivalents, each one of which is formed by four chromatids (Fig 81, 4) The anaphase of the first meiotic division (Anaphase I) brings about the separation of the homologous chromosomes, which pass to the respective poles of the cell. After a short period of interkinesis, the second meiotic division takes place (Fig 81, 6) Anaphase II (Fig. 81 7) brings about the separation of the longitudinal halves or chromatids of each one of the homologues. From this moment each chromatid should be considered as a chromosome passing to each pole.

In summary the first meiotic division separates the homologues which have been paired, while the second meiotic division separates their longitudinal halves. After the two meiotic divisions there are four cells, each with the haploid number of chromosomes.

The time at which meiosis occurs during the life cycle varies with different organisms but is constant for each particular species. Three types may be distinguished.

(1) *Initial or syzyotic meiosis* which takes place immediately after fertilization, at the beginning of the cleavage of the egg. As a consequence, an adult haploid individual results. This is the most primitive form of this process and occurs in certain fungi (Basidiomycetes, Ascomycetes) in certain Algae and in some Sporozoa.

(2) *Intermediary meiosis or by spores*, which takes place during the formation of the spores, between the stages of the sporophyte and the gametophyte. This is common among some thallophytes and in higher plants. In the latter a longer phase predominates, that of the sporophyte, which is diploid during its whole existence. The gametophyte period is very short and haploid. In the lower plants the gametophyte predominates and the organism is haploid. Among the mosses and ferns fertilization alternates with meiosis, sporophytes originate from the zygote and produce spores, while gametophytes originate from spores and produce gametes.

In the angiosperms, meiosis begins in the female gametophyte with the division of the mother cell of the embryo sac (megaspore). This is followed by the second meiotic division. In some plants a third division occurs, mitotic in character which gives rise to eight nuclei: the ovule or oosphere, two synergids, three antipodal and two polar nuclei. These last fuse later to contribute to the nucleus of the endosperm. It follows from this that of all these cells only one, the ovule, will become a mature sex cell. Microsporogenesis is simpler. Since each one of the pollen mother cells undergoes the first and second meiotic divisions, four nuclei result, each one with the haploid number of chromosomes. In animals, the corresponding four nuclei develop into four spermatozoa. In plants, each one of these nuclei forms a grain of pollen, which again divides by mitosis (during the formation of the pollen tube) giving one vegetative and one germinative nucleus. This last divides again and produces two antherozoids.

(3) *Terminal or gametic meiosis* which is common to all Metazoa and is found in some Protozoa and lower plants (thallophytes). This meiosis takes place

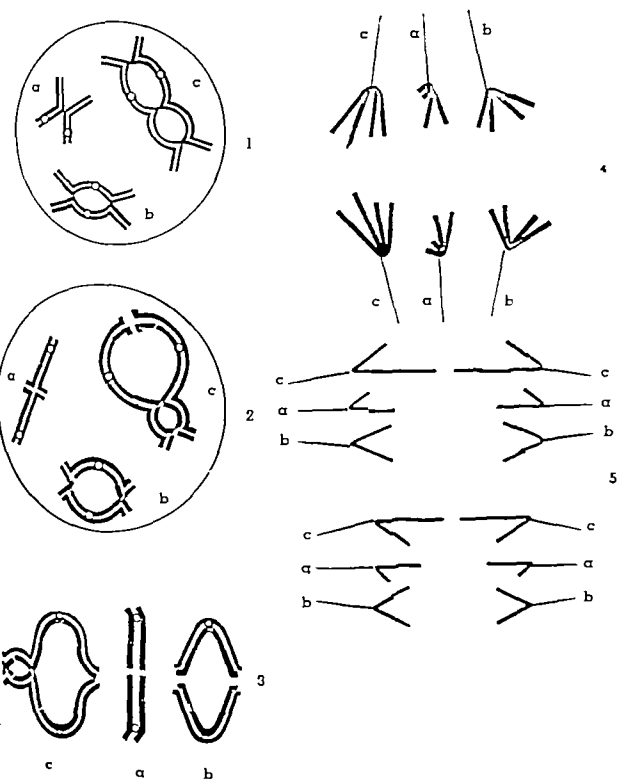


Fig. 8. Diagram showing the genetic consequences of the meiosis of three pairs of chromosomes with (a) one chiasma (b) two chiasmata and (c) three chiasmata. 1 diplotene 2 advanced diplotene showing the process of terminalization 3 metaphase I 4 anaphase I 5 anaphase II showing the distribution of the chromosomes in the four nuclei formed. In red, the paternal chromosomes and in blue the maternal. The centromere is indicated by a circle.

during gametogenesis (spermatogenesis and oogenesis) In animals, germ cells originate from *initial cells*, which, by division, give rise to *somatic* and *germinal* cells. Germinal cells, by repeated divisions, produce many generations of *primordial* cells, cytologically undifferentiated, the aspect of which is similar to that of any embryonic somatic cell. After a quiescent period of variable duration and after the gonads have been formed, these primordial germ cells usually are transformed into *gonial* cells. These are initially very similar in both sexes, but afterwards differentiate into primary spermatogonia and primary oogonia. Later by division of the primary gonial cell, the secondary *gonia* (spermatogonia and oogonia) develop. Each secondary spermatogonium gives rise, in a final division, to two daughter cells which begin to increase in volume and are called *primary spermatocytes* or *spermatocytes I*. Growth takes place during the whole prophase and metaphase I. At division the primary spermatocyte (*primary meiotic division*) gives rise to two daughter cells or *secondary spermatocytes* which divide again (*secondary meiotic division*) resulting in four cells or *spermatids*. These cells, by differentiation (spermiogenesis or spermatelions) are transformed into spermatozoa. In the female the successive stages are *oogonia*, *primary oocytes*, *secondary oocytes*, *ootids* and *ova*, with the difference that, in place of four functional gametes there is only one, the mature ovum, since the other three are infertile *polarocytes* or *polar bodies*.

### Analysis of Meiosis

The meiotic process begins very early and, in some animals, is found even in the embryonic stage. The classical stages of mitosis do not suffice to describe in a complete manner the development of the stages and the complex movements of the chromosomes in meiosis. These stages may be listed in their successive order as follows (Figs 81 82 83)

- |          |                     |
|----------|---------------------|
|          | Preleptotene stages |
| Prophase | Leptotene           |
|          | Zygotene            |
|          | Amphitene           |
|          | Pachytene           |
|          | Diplotene           |
|          | Diakinesis          |
|          | Prometaphase I      |
|          | Metaphase I         |
|          | Anaphase I          |
|          | Telophase I         |
|          | Interkinesis        |
|          | Prophase II         |
|          | Metaphase II        |
|          | Anaphase II         |
|          | Telophase II        |

*End of the Resting Period and the Preleptotene Stages* In the final stage of the somatic telophase of the gonia the chromosomes continue to be arranged in the same way as in the anaphase.

There are cases in which the chromosomes cannot be seen, due to the extreme delicacy of the chromonemata. This stage is analogous to the early prophase of mitosis. Analysis of this stage is difficult when there are no sex chromosomes present, as their differential heteropyknotic behavior aids in the interpretation of the course of this phase (Fig. 83, 1, 2, 3). The telophasic disintegration occurs while the spirals are slowly loosening. In certain insects the individuality of the chromosomes is not lost, since they are found spirialized inside vesicles or compartments which lodge each chromosome separately (Fig. 83, 1 and 2).

*Leptotene Stage* (Figs. 81, 1 and 83, 4). While the chromosomes become apparent with greater clarity as long filaments well separated from each other, they begin to show the chromomeres, which look like the beads of a rosary. So precise and constant are the locations and sizes of these chromomeres, that they may serve to identify some particular chromosome.

The number of chromosomal filaments is equal to the somatic number and may be counted without difficulty in nuclei with a few chromosomes.

The arrangement of the leptotene chromosomes is often irregular but they may lie with a definite orientation and a tendency to be polarized, directing one or both ends toward the nuclear membrane, commonly toward the point where the centrosome is located (Fig. 83, 5).

When polarization of the ends of the chromosomes occurs they are arranged in a parallel and convergent form, being found close together at the point of polarization while the distal ends spray out in the nuclear cavity. This peculiar arrangement has received the name of "bouquet" (Fig. 83, 6). The filaments that are still moniliform approach each other anew coming into lateral contact.

*Zygotene Stage* (Figs. 81, 2 and 83, 6, 7, 8, 9). At the beginning of this stage the union of homologous chromosomes starts. The arrangement during the pairing is varied. Sometimes the chromosomes unite by their polarized ends and the pairing goes on to the antipodal extremity. In other cases the fusion takes place simultaneously at various places along the length of the filament. The presence of a bouquet and polarization in general seems to favor regularity in pairing. The zygotene pairing is customarily called *synapsis* or *syndesis*, although these terms are falling into disuse.

The attraction of the homologues and their later conjugation are very significant and constitute an important part of meiosis. The pairing is remarkably exact and specific. It takes place point for point, chromomere for chromomere, of each one of the homologues. The attraction apparently results from a property which each chromosome has of uniting with another to constitute pairs.

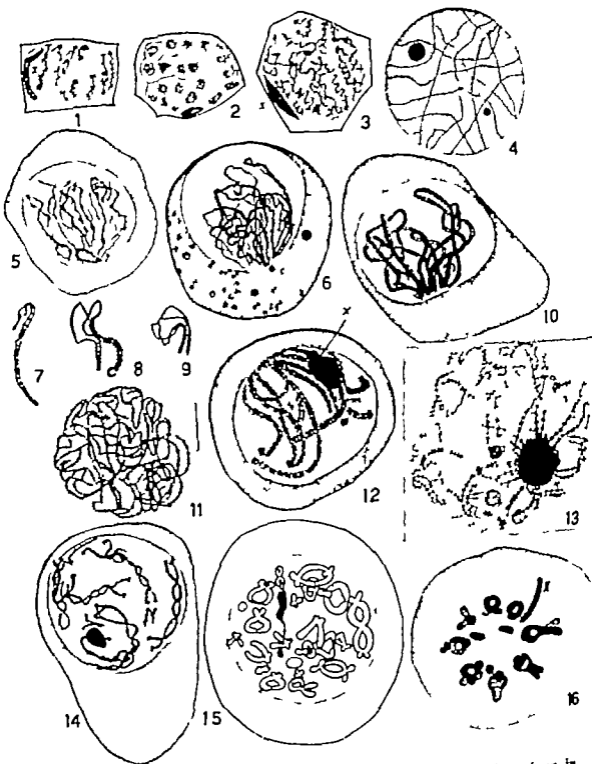


Fig. 83. Various typical stages in meiosis. 1 Last gonial telophase (seen in profile) of the locust *Phrynotettix*, showing the chromosomes (including the sex chromosome (X)) arranged in a parallel manner; 2 frontal section of the same stage; 3 preleptotene stage in the same animal; 4 leptotene stage in the toad *Bufo arenarum*; 5 polarization of the chromosomes during the leptotene stage. The element which is seen below against the nuclear membrane is composed of the sex chromosomes X and Y (red weasel, *Lutreolina crassicaudata*); 6 zygotene stage in the planaria *Dendrocoelum* arranged in "bouquet" formation; 7 pairing of the

of genes and to begin their interchange. On the other hand, we recall that during the zygotene stage the chromosomes consist of a single undivided filament. In this condition it seems to have attractive forces which will be satisfied or neutralized only when pairs of homologous filaments are formed \*

*Amphitene Stage* This represents a stage of equilibrium of short duration, characterized by a nonuniform appearance of the chromosomes. It is especially evident when association is carried out between polarized filaments. Under these circumstances parts of the chromosomes are found closely united, forming heavy double cords, while the opposite extremes are still free (Fig 83 8)

*Pachytene Stage* (Figs 81 3 and 83 10 11 12) When the longitudinal pairing of the leptotene chromosomes is completed, a longitudinal contraction takes place which results in the formation of shorter and coarser filaments. By the aid of refined techniques a double constitution of the filament can generally be observed (Fig 83 10 11 12). At the height of the pachytene stage, the nucleus is seen to contain a group of double filaments, haploid in number. From this time it contains half the full number of chromosomes, but this reduction is only apparent since each chromosome is double and is composed of two homologues in close longitudinal union. In each chromosome the double series of chromomeres can be seen paired one for one. In some cases the correspondence existing among the different chromomeres has been demonstrated not only in the individual, but also among the species of a particular genus. Belling has demonstrated the uniformity, constancy of size, structure and location

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homologues 8 intermediate, amphitene stage during zygotene pairing; 9 another step in the process of pairing; 10 pachytene stage showing the double constitution of the bivalents (all these figures are from *Dendrocoelum*); 11 pachytene stage in the iris showing the chromomeres which reach the number of 2193; 12 polarized pachytene stage in the locust *Schistocerca gregaria*, showing the X chromosome in positive heteropyknosis; 13 beginning of the diplotene stage in *Trillium erectum*; 14 middle diplotene stage in the red weasel, showing the chiasmata of each bivalent, as well as the nucleolus in the upper part and the sex chromosomes in the lower part of the nucleus; 15 terminal diplotene in the human species, showing the chiasmata and the sex chromosomes X and Y; 16 metaphase I (in polar view) in the locust *Schistocerca*. The sex chromosome has less nucleic acid (negative heteropyknosis) while the other chromosomes show their various configurations and the local position of their chiasmata (1-3 After Wenrich, 1916; 4 after Saez, Rosa and De Robertis, 1930; 5 after Saez unpublished; 6-8 9 10 after Geley, 1911; 11 after Belling, 1929; 12 after Saez, 1927; 13 after Huskins and Smith, 1935; 14 after Saez, 1931; 15 after Koller, 1933; 16 after Saez, 1930)

In the description of the leptotene and zygotene stages the chromosome is considered to be formed by a single filament. However, cases of preleptotene splitting have been described (Huskins, Robertson).

of the chromomeres distributed along the pachytene chromosome and, in some liliaceous plants, has counted 1500 to 2500 chromomeres (Fig 83 11) It is a common finding that when the pachytene chromosomes are long they twine among themselves, due to a torsion of the homologous filaments, thus constituting a relational spiral between them. Each chromosome forming the double pachytene element has its independent centromere so that each bivalent has two centromeres. These are

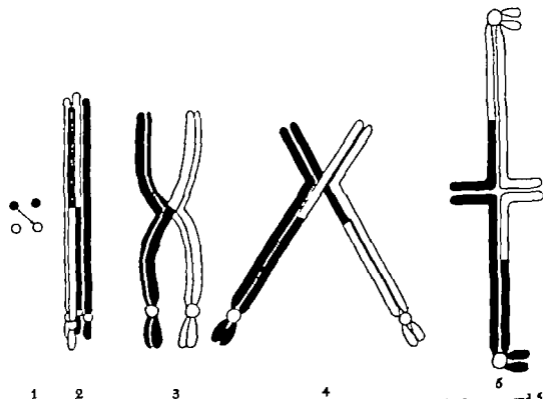


Fig. 84 Diagrams of 1 and 2 crossing over; 3 chiasma; 4 terminalization and 5 rotation of the chromatids of a bivalent. The circles indicate the centromeres.

found very close together. About the middle of the pachytene stage there occurs a longitudinal cleavage in each homologue in a plane perpendicular to that of the pairing. At this time a bivalent is composed of two homologous chromosomes, united throughout their whole extent but each one separated into two chromatids. This means that at this stage of the prophase each pachytene element consists of four chromatids (Fig 81 3c). The chromatids of each homologue are called *sister chromatids*. Formerly this element composed of four chromatids was called a *tetrad*, a name which has been replaced by that of a *bivalent*.

Almost simultaneously with the longitudinal cleavage of each chromosome, transverse breaks may occur at the same level in two of the homologous chromatids. Immediately afterwards, both

segments of the chromatids interchange and fuse together with those of the homologues (Fig 84, 2) In this process called cross ing over portions of two chromatids with their genes are inter changed and two remain intact. The interchange never is carried



Fig 85 Behavior of the bivalents during the diplotene stage A and the metaphase, B C D E, showing the position of the chiasmata. The total number of the chiasmata of each chromosome along with the number of terminal chiasmata is indicated below the respective chromosome, the former with the larger figure, the latter with the smaller. The sums of the total chiasmata and of the terminal chiasmata for each nucleus are indicated at the right of each row in the form of numerator and denominator respectively. The sex chromosome is indicated by X (*Schistocerca gregaria*) (After Seitz, 1938.)

out between daughter chromatids, only between homologous chromatids. The new chromatid thus interchanged will be mixed each will have segments of the other (Fig 84 5).

**Diplotene Stage** (Fig 81 1 82, 1 83 11 15 84 3) After the longitudinal cleavage of each homologous chromosome they

start to separate, repelling each other. However this separation is not complete, since the homologous chromosomes remain united together by their points of interchange (crossings over) which are called *chiasmata* (Figs 82 1 83 14, 15 and 84, 3). At the chiasmata the chromatids are held together as if by a knot. With few exceptions, chiasmata are found in all plants and animals. At least one chiasma is formed for each bivalent. Chiasmata are generally interstitial, that is to say they are formed in between the

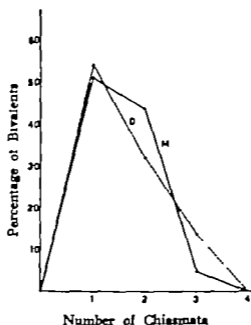


Fig. 86. Curves of the comparative frequency of chiasmata in the diplotene, *D* and in the metaphase, *M* of the bivalents of *Schistocerca gregaria* made with the data taken from Fig. 85. (After Saex, 1938.)

ends of the chromosomes (Fig 82 1). Their number is variable, since there may be chromosomes which have one chiasma and others which possess up to thirteen

The number of chiasmata is generally proportional to the length of the chromosomes, but there are exceptions. Thus in the hyacinth and in some orthopteran insects the short chromosomes have almost the same number of chiasmata as the long ones (Figs. 85 and 86). It has been suggested that this may depend on genetic adaptation (Darlington, 1937). Time may also play an influential role during pairing. When the chromosome is long, the pairing begins in the region of the centromere and is lacking in the more distal regions. In such cases, chiasmata are formed in the paired segments and in the region of the centromere. Short chromosomes presumably have more time for pairing. Short subtelocentric chromosomes have a greater number of chiasmata than metacentric chromosomes. In these small chromosomes chiasmata are localized at the distal ends. Such chromosomes lend themselves more favorably to a mechanical displacement.

During the diplotene stage the chiasmata in most species begin a movement tending to reduce their number in each bivalent. The movement consists in a displacement of the chiasmata along the length of the chromosome from the centromere toward the ends. This displacement of the chiasmata was designated

*terminalization* (Darlington, 1929) (Figs. 82, 2 84-4 and 85) During the course of the diplotene stage there is produced a contraction and thickening of the chromatids due to the winding up of the internal spiral of the chromonemata. In

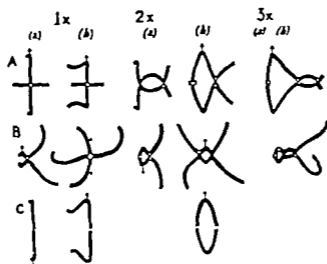


Fig. 87 Diagram of the configuration of the bivalents during metaphase I showing the position of the chiasmata. A, With nonterminalized chiasmata. B With nonterminalized chiasmata, but with localization. C, With completely terminalized chiasmata. (a) and (b) Subterminal and median centromeres indicated by the arrow  $1x$   $x$   $3x$  Respective numbers of the chiasmata. (After Darlington, 1937)

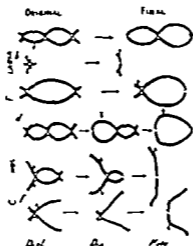


Fig. 88 Diagram of the various types of terminalization. a, b, c The type seen in *Fritillaria*, with little terminalization, change in position and diminution in number of the chiasmata. d, e, f type seen in *Campanula*, with terminalization of all of the chiasmata. These are in the way of completing the process. (After Darlington, 1937)

essence this longitudinal contraction of the chromatids is similar to that which occurs in mitosis (page 179)

At the same time there occurs a third movement called *rotation*, which seems

In certain exceptional cases the chiasmata are maintained in almost the same original position from the diplotene stage up to the diakinesis and beginning of the metaphase (Figs. 87, 85 and 89)

to result in part from the same force of repulsion to which is attributed the divergence of the homologous chromosomes. This rotation is more obvious when the bivalent has a single chiasma. Figure 84 4 5 shows the arms of the bivalent executing a movement which consists of a rotation of 180 degrees until they acquire the form of a cross (Figs. 82, 2a and 84 5). If there are two chiasmata, the openings which occur between them continue to widen until a ring is formed. If there are more than two, the rotation gives rise to a figure 8, or to a chain of links each one of which is perpendicular to the next. These different movements give rise to the classical configurations of the bivalents in the form of V, 8, X, O, + found at the end of the diplotene and in diakinesis (Figs. 82, 1 88 and 89). Upon completion of the



Fig. 89 Metaphase I in polar view in *Fritillaria* showing the chromosomes with numerous chiasmata (the number of which is indicated in each bivalent) (After Darlington, 1930.)

terminalization of the chiasmata, the homologues are maintained in contact by terminal chiasmata (Figs. 84c, 85 and 88c, f). Long chromosomes generally have a partial or very limited terminalization. *Tradescantia virginiana* constitutes an exception since, in spite of the fact that it has very long chromosomes, it shows distinct terminalization (Darlington, 1929; Richardson, 1935). The degree of terminalization appears to depend upon the repulsions between the chromosomes and between the centromeres, and upon the length of the chromosomes. There exists also a certain correlation between the degree of contraction and the terminalization of the chromosomes. There are cases in which the terminalization is interrupted due to a change produced in the homology of one of the chromosomes at a particular region. This occurrence impedes pairing. Such a happening may indicate a disharmony between the homologues. In the last instance, the localization, terminalization and distribution of the chiasmata in the metaphase are subject to the operation of the genotypic control. The degree of terminalization may be expressed as a coefficient of terminalization (T)

$$T = \frac{\text{Number of terminal chiasmata}}{\text{Total number of chiasmata}}$$

The average number of chiasmata which are found in a bivalent or in all the bivalents of a nucleus constitutes the frequency of chiasmata (Fq)

To understand better the various motions which succeed one another during meiosis and to visualize the spatial relationships of the chromatids, it is often helpful to use a plastic material, cords, or wires and to model the forms which arise at the various stages.

$$Fq = \frac{\text{Total number of chiasmata}}{\text{Total number of bivalents (or of nuclei)}}$$

The graphic expression of this is a curve such as that shown in Figure 86

*Diakinesis* The transition from the diplotene stage to that of diakinesis takes place gradually. Diakinesis is characterized by an accentuated contraction of the chromosomes which is even greater than that found in the final stage of the prophase of mitosis. The traces of splitting which persisted during the middle and final phases of the diplotene stage disappear. The arrangement of the chromosomes in the nucleus is subject to the same conditions seen at the end of the prophase of mitosis and depends on the relation existing between the size of the bivalents and the volume of the nucleus. The nucleoli behave much as in mitosis. Meanwhile, the process of terminalization continues while the number of interstitial chiasmata diminishes. The chromatids remain connected by the chiasmata and are maintained until the metaphase. When terminalization is completed, the members of the pair of homologues remain in contact only at their distal extremities where the centromeres are located. The condensation advances along with the linear contraction, which now becomes pronounced. Thus *prometaphase* is reached, the characteristics of which are similar to those of the same stage in mitosis. Spiralization reaches its virtual maximum, the nuclear membrane disappears and the chromosomes become arranged on the equator of the cell to begin the metaphase.

*Metaphase I* (Figs 82, 3, 83, 16 and 89) The bivalent chromosomes arrange themselves in the equatorial plane so that the two members of each homologous pair are found with their centromeres directed toward opposite poles (Figs 82, 3 and 84, 5). While in mitosis there is a single centromere which unites the chromosomes, in meiosis there are two centromeres and these occur separated from each other at the time of the metaphase. Each homologous chromosome has here a centromere to which the daughter chromatids are united. We may recall that the form of the metaphase chromosomes depends on the location of the centromere, on the size of the chromosomes and on the number and position of the chiasmata. The repulsion of the centromeres is accentuated and the chromosome is found on the verge of dividing. If the bivalent is long it presents a series of annular apertures between the chiasmata in planes alternating perpendicularly among themselves. If the chromosomes are short, they have a single aperture of annular shape or they exhibit the form of a shaft, rod, or sphere (Figs. 83, 16 and 85).

In certain cases there is a *third splitting* by which the bivalents come to be constituted of a double chromonema in each of their chromatids. In such instances each bivalent presents a structure composed of eight filaments instead of four. It is presumed that this separation is produced submicroscopically at an earlier time, but it comes into evidence at the end of the diplotene stage or in the metaphase and anaphase I and II. It is best observed in the mitoses in the pollen grain (prophase of the first mitosis of the pollen grain or microspore, see Fig 70, *b*). This last division should not be confused with the meiotic divisions I and II, since it actually corresponds to a postmeiotic mitosis.

*Anaphase I* The daughter chromatids, united by their centromere, move toward their respective poles. The short chromosomes, generally connected by a terminal chiasma, separate rapidly. The long chromosomes, with interstitial and unterminalized chiasmata, are delayed in their separation. While the terminalization of these chromosomes proceeds, the chromatids separate gradually as if overcoming the resistance of some adhesive substance binding them together. This is explained as being due to the resistance of the chiasmata as they are passing to the end of the chromosome. For this reason, long chromosomes are delayed in their way toward the poles. In profile, anaphasic chromosomes show different shapes which depend upon the position of the centromere.

It should be recalled that by virtue of the formation of the chiasmata, a transference of segments was produced between the two chromatids of each homologue. Thus when the homologous paternal and maternal chromosomes separate in the anaphase, they have a different composition from that of the originals. Two of their chromatids are mixed, while the other two maintain their initial nature (Figs 81, 5 and 82, 4). Each centromere is directed toward a different pole, the maternal centromere to one and the paternal centromere to the other.

*Telophase I* The first manifestations of the telophase begin as soon as the anaphasic groups arrive at the respective poles. These are the reconstruction of the nuclear membrane and the transformation of the chromosomes. The latter is, however, not always very marked. Chromosomes may persist in a condensed state showing all of their morphological characteristics. In this case the two daughter chromatids diverge from each other markedly as if mutually repelling, and acquire the form of V or X (Fig 81, 6). Following the telophase there is a short period called *interkinesis* which has characteristics similar to the interphase of

The ends of the chromosomes have, in some cases, a certain tendency to cohere (Hughes-Schrader 1945; Hinton, 1945).

mitosis (Fig 81, 6) The result of this division I is the formation of the daughter nuclei, which in animals are called spermatocytes II (in the male) and oocytes II (in the female)

*Prophase II* After the interkinesis a short prophase takes place, followed by the formation of the achromatic spindle which marks the beginning of the next stage.

*Metaphase II* If we observe the chromosomes in polar view during this metaphase, we find that their number is half that of the somatic number The chromosomes become arranged on the equatorial plane, the centromeres divide and the daughter chromatids direct themselves toward the opposite poles (Fig 81, 7) Since in this division the longitudinal halves of each parental chromosome (chromatids) separate, each one of the four nuclei of telophase II will have one chromatid which now is called a chromosome Each nucleus has a haploid number of chromosomes and one complete genome, in which each chromosome is represented once (Figs 81 8 and 82 5)

*The essence of the meiotic process* is seen in the formation of four nuclei, each differing from the others, in which each chromosome of the parent is represented once. The total number of chromosomes is not actually "reduced," neither in the first nor in the second division, but the final result of the two divisions is the distribution of the chromosomes. The older concept of reductional and equational divisions, by which the homologous chromosomes are separated (or segregated) in the first division and in the second, the halves of the same are separated, cannot be strictly applied in the light of modern knowledge concerning the phenomenon of crossing over (see below) In Figure 81 chromosome B is effecting a "reduction" in anaphase I and an equational division in anaphase II. On the other hand, the segment located between the distal end and the chiasma of chromosome A is effecting a "reduction" division in anaphase II. A single chromosome may thus carry out a reductional or an equational division in either one of the two divisions (Fig. 82, 4 5) Each chromosome may separate independently of the rest of the group and direct itself, with equal probability toward either one of the two poles.

Meiosis should be considered as a profound modification of mitosis by an alteration of the time relationships at which the chromosomes pair and divide longitudinally The prophase is more precocious in meiosis and, therefore, the homologous chromosomes accomplish the pairing before they become double (Darlington) Meiosis is a mechanism for distributing the hereditary units or genes, permitting their fortuitous independent recombination. Crossing over provides a means whereby genes which are found in different chromosomes can be brought together and recombined If this process did not take place the evolution of the species would be suspended by unalterable chromosomes and living nature would not have its characteristic diversity

## THE CHROMOSOMES AS THE MATERIAL BASIS OF HEREDITY

The tendency of offspring to resemble progenitors is called heredity Yet offspring do not resemble their progenitors exactly This common tendency in plants and animals to show dissimilarity gives importance to the study of heredity A child may

in some respect resemble its father in another its mother or even an ancestor, although it resembles no one of them in all respects. Present day biology is able to explain in part the causes which have determined the production of such different characteristics in progeny.

In 1865 Gregor Johann Mendel, while studying the crosses between peas (*Pisum sativum*) discovered the laws which rule the mechanism of hereditary transmission. A long series of experiments, continuing up to the present day have demonstrated that these laws of heredity are widely applicable in the biological world.

In order to understand these laws better let us take the case of a cross between two different varieties of the species *Mirabilis jalapa*, one with white flowers and another with red flowers. The generation which results from this cross is called  $F_1$  (first filial generation) and consists exclusively of pink flowers. These individuals crossed among themselves give [in the following generation called  $F_2$  (second filial" generation)] red, pink, and white flowers in the proportion 1 2 1 (or 25% 50% 25%) (Fig 90) The plants with red flowers and those with white, when crossed among themselves, produce respectively red and white flowers. On the other hand when the pink flowers are crossed with each other they give rise to a generation with the same proportion of colors as in  $F_2$  (1 2 1), and so on.

The experiments of Mendel on peas seemed much more complicated than the crosses occurring in *Mirabilis*. Crossing, for example, a dwarf with a giant plant, Mendel obtained in the  $F_1$  generation giant plants exclusively and in the  $F_2$  generation plants of different heights, but in a fixed proportion of three giants for one dwarf plant (that is, 75 per cent and 25 per cent) The crossing of the members of this generation among themselves showed that the dwarf plants always produced dwarf individuals (just as in the case of the plants with red or white flowers in *Mirabilis*) but the giant plants in succeeding generations gave the same proportion of giant and dwarf plants as in the  $F_2$  generation (3 1)

To explain these facts, Mendel supposed that within the sex cells (gametes) there should exist a factor corresponding to each visible (phenotypic) character. When a giant plant is crossed with a dwarf, in the individuals of the  $F_1$  generation the factors of gigantism and of dwarfism are joined together (for this reason they are called *hybrids*)

This last character (dwarfism) although it remains hidden, actually is found in the gametes of the hybrid, as demonstrated

by the fact that in succeeding generations dwarf plants are produced in a fixed proportion. Mendel arrived at the conclusion that in these gametes the factor giant (a *dominant* factor) dominates the factor dwarf which is hence called *recessive*

In the crosses which Mendel made he was able to prove that, as a consequence of dominance, the relationship 3 : 1 pertains only to the visible or phenotypic character. Indeed when the experiments were followed in the  $F_2$ ,  $F_3$  and other generations, there was found among the giant plants a fixed number (25 per cent) of tall plants which always produced giant plants with this same characteristic. These plants correspond to the tall plants of the first generation (called parental P) and can be compared with the red or white plants of the  $F_2$  generation of *Mirabilis*.

Until now only the visible characteristics (phenotypic characteristics) of the crosses had been studied. In order to explain the intimate mechanism of this process, Mendel theorized that the hereditary units of these opposed characteristics (*alleles* or *allelomorphs* as we now call them) are localized in the gametes and are separated in the hybrids. In this way upon the formation of the sex cells, the hereditary units are distributed among them in identical proportions (first law of Mendel). In the fertilization which follows this separation the hereditary units are recombined according to the laws of chance and, in this way, new combinations and recombinations are formed, the number of which depends upon the characters which enter into the cross (second law of Mendel). We now call these hereditary units *genes*.

Considering again the crossing of *Mirabilis jalapa*. The white and the red flowers have gametes where the *gene*, white or red, is represented in double quantity (one from the female parent and the other from the male). In the diagram (Fig. 90) these factors or genes are represented by a large circle with a double R or a double r respectively. After the process of meiosis, all of the gametes will contain only one factor R or r. From the union of the sex cells containing R, with those containing r a plant originates with the genetic constitution Rr. Since these characteristics are *intermediary* plants will develop with the phenotypic character of possessing pink flowers. This type of flower has two types of hereditary factors and for this reason is called *heterozygous*. In the course of the production of the new gametes these factors are separated and two types of sex cells are formed, 50 per cent of which contain the factor R and 50 per cent of which contain the factor r. To this process one can apply the first law of Mendel.

When hybrids of this type are crossed, there are four possibilities (according to the laws of chance) for combinations (see Fig 90)

RR Rr rR rr

Of these combinations the first and last are *homozygous*, while the other two are *heterozygous*

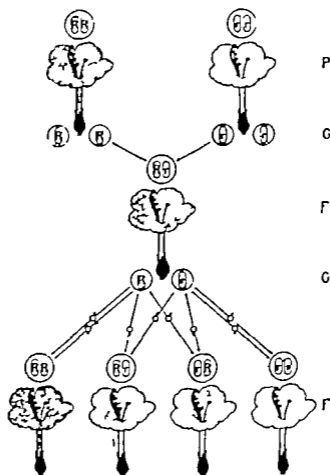


Fig. 90 Diagram of mendelian segregation in *Mirabilis jalapa* (red and white flowers) The gametes are indicated by small circles, the genotypes by large circles (After Bělár)

When more than two characters are crossed, the number of possible combinations increases. Thus, for example, crossing peas having yellow and smooth seeds with peas having green and rough seeds yields, in the F<sub>1</sub> generation, hybrids with yellow (dominant in relation to green) and smooth (dominant with respect to rough) seeds. When these hybrids are crossed (in the F<sub>2</sub> generation) not only homozygous yellow-smooth and green rough seeds, but also yellow rough and green-smooth (these last two heterozygous) in the following ratios

9 Yellow Smooth (homozygous)	1	3 Yellow Rough	1	3 Green Smooth	1	1 Green Rough (homozygous)
heterozygous						

To these processes one can apply the second law of Mendel, since the gametes of the hybrids became united, forming all of the possible combinations. In the case of more than one pair of characters new combinations are formed, as in the appearance of yellow rough seeds and green-smooth seeds.

In summary the laws of Mendel may be defined as follows:

1 *Law of the separation or segregation of the hereditary units* The hereditary units which represent opposed characters in a cross are separated in the hybrid at the formation of the sex cells and are distributed among these latter in identical proportions.

2 *Law of independent combination* The hereditary units, separated during the formation of the gametes, unite again in the following generation according to the laws of chance and form new combinations and recombinations, the number of which depends on the number of the characters which enter into the cross.

In the following table are given the gametic and zygotic combinations in accordance with the number of pairs of factors in the hybrid.

TABLE IX  
GAMETIC AND ZYGOTIC COMBINATIONS IN ACCORDANCE WITH NUMBER OF PAIRS OF FACTORS IN THE HYBRID

Number of Pairs of Factors	Number of Gametes, Different Classes in Equal Number	Number of Zygotic Combinations in the F <sub>2</sub>
1 Aa	2 A, a	4 AA, 1
2 AaBb	4 AB Ab, B, b	16 9AB 5Ab, 3aB 1ab
3 AaBbCc	8 ABC, ABc, AbC, aBC, Abc, Bc, abC, bc	64 27ABC 9(ABc, AbC, BC) 3(Abc, aBc, abC) 1 bc
4	16	256
5	32	1024
10	1024	1 048 576
24	16 777 216	281 4 427 680 10 636
n	2 <sup>n</sup>	4

On November 18, 1901, after the appearance of the works of Correns, Tschermak and de Vries, in which they confirmed the famous results of Mendel, Montgomery (1901) (who was not aware of the postulates of Mendel) found that during the formation of sex cells the paternal and maternal chromosomes became united in pairs (conjugation).

before being separated. The parallelism between the mechanism of the Mendelian factors and the chromosomes was, however definitely established by the publication of three classical works, in which evidence was given of the morphological and physiological differences of the chromosomes. Boveri and McClung, in 1902, independently showed their functional differences and Sutton (1902) demonstrated their morphological characteristics.

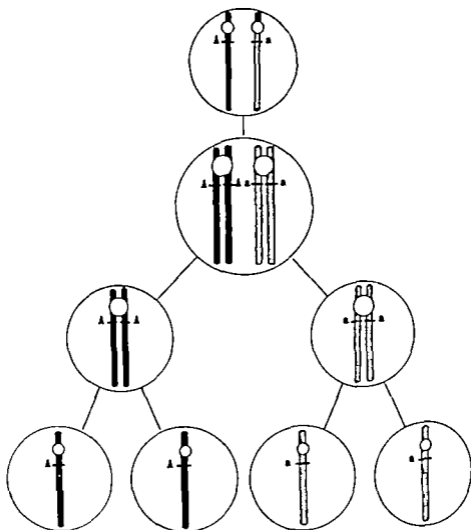


Fig 91 Diagram of the segregation of a pair of genes localized in a pair of chromosomes without crossing over occurring during meiosis. Two classes of gametes result

Boveri, studying larvae of the sea-urchin in which an experimental alteration of the normal combination of the chromosomes was produced by means of a double fertilization, reached the conclusion that a given combination of chromosomes was essential in order to complete the normal development of an individual, but that a definite number of chromosomes was not necessary. McClung set forth the hypothesis that the accessory unpaired chromosomes (page 242) studied by him in the locusts, was the sex determinant and suggested for the first time the relation between a particular chromosome and a specific character. This interpretation is the precursor of that proposed years later by Morgan, who conceived the idea of sex-linked hereditary factors from the alternating distribution of the sex chromosome (page 220). Sutton demonstrated that the chromosomes of biparental origin were homologous and that they united in pairs, later to be separated and to be incorporated into each germ cell so that each one of these contains one chromosome of the pair.

This important observation made evident the parallelism between Mendelian segregation and the segregation of the biparental chromosomes. These discoveries laid the foundations for the chromosome theory of heredity.

As we have seen, the mechanism which explains the segregation of the genes is the pairing of the homologous chromosomes and their later separation, each one to form part of the newly formed cells. If in the crossing of a pair of allelomorphs, the dominant gene (A) were in one chromosome and its recessive allelomorph (a) in the respective homologue, the method of distribution seen in Figure 91 would occur

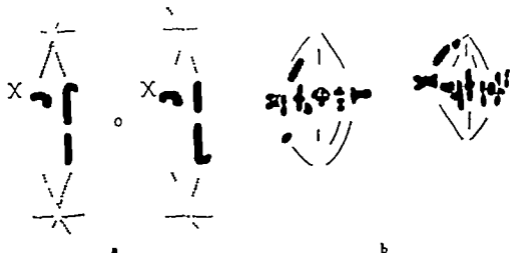


Fig 92. Segregation according to the laws of chance of the chromosomes during the first meiotic division. *a*, a heteromorphic (unequal) pair showing their behavior with respect to the sex chromosome indicated by X. *b* behavior of a chromosome with regard to the sex chromosome which is passing toward the opposite (left) and in another case toward the same (right) pole as the latter (*a*, After Carothers, *b* after Saez, 1931)

We owe to Carothers a series of works which very well demonstrate the fortuitous segregation of the chromosomes. Due to the fact that in locusts of the genera *Circotettix* and *Trimerotropis* some pairs of chromosomes exist in which the homologues are different (heteromorphic) (one in the form of J and the other of I) she was able to study in detail the passage of each one of these elements to the different poles during the first part of the anaphase. She took as a point of reference the sex chromosome which is unpaired and found that in 300 anaphases the larger homologue of the heteromorphic pair passes to the same pole as the sex chromosome in 51.3 per cent of the cases. This agrees very well with the theoretical distribution which would predict that this chromosome would pass to either pole 50 per cent of the distribution (Carothers, 1936) (Fig 9-*a*). In the locust *Orphulella punctata* one chromosome which travels ahead of the others in the first part of the metaphase passes sometimes to the same pole as the sex chromosome, and at other times toward the opposite pole (Saez, 1931) (Fig 9-*b*).

If in table IV, instead of pairs of factors, we had pairs of chromosomes which are freely distributed, we would have a clear example of the mechanism of segregation. Figure 93 illustrates this mechanism in the case of four pairs of chromosomes distributed according to chance and their respective combinations in each one of the sixteen gametes formed, as is seen in *Drosophila melanogaster*.

### Linkage and Crossing Over

Intensive study and experimentation on Mendelian heredity led to the view that in many cases there is not absolute independence of the factors located in the chromosomes and that

there is a certain limitation to the free segregation of some factors. It was seen that in such cases there is a marked tendency for parental combinations to remain linked and that a lesser proportion of new combinations occurs. The phenotypic proportion differs from that which is to be expected in accordance with the second law of Mendel.

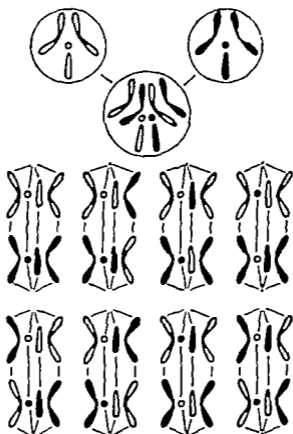


Fig. 93 Diagram of the distribution according to the laws of chance of the chromosomes during oogenesis of *Drosophila melanogaster*. Above, the gametes and the zygote. Below the 8 types of distribution which will give rise to 16 classes of gametes. In white, the maternal chromosomes and in black, the paternal. (After Babcock and Clausen, 1937.)

If for example, one crosses a dihybrid with a double recessive, four combinations may appear in the proportion 1 1 1 1. This cross can be represented

$$\begin{array}{c} AaBb \times aabb \\ 1 ABab \quad 1 Abab : 1 aBab : 1 abab \end{array}$$

It is interesting to point out that the progeny of this cross corresponds, in its aspects and proportions, to the classes of gametes produced by the hybrid father. Now if the two genes (A and B, or a and b) are in the same chromosome instead of in different chromosomes, the result of a cross of a dihybrid with

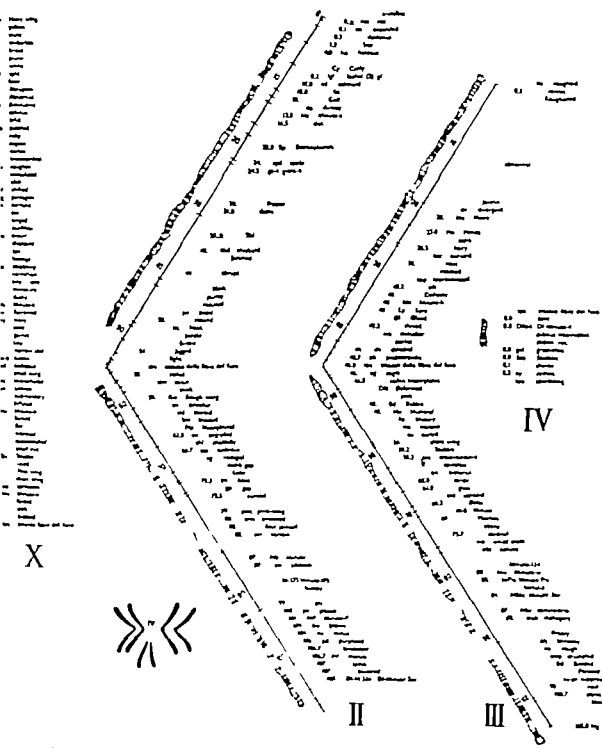


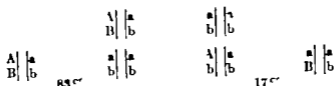
Fig 94 Genetic map of the four chromosomes of *Drosophila melanogaster* together with the cytologic map of the polytenic chromosomes. Below and at the left the normal somatic chromosomes of the male are represented (After Timofeeff-Resovskiy, 1939)

the recessive will be different. In this case there appear only two combinations in the proportion 1 : 1, each corresponding to one of the parents, thus

$$\begin{array}{rcl} \text{ABab} & \backslash & \text{abab} \\ 1 \text{ ABab} & & 1 \text{ abab} \end{array}$$

Figure 96 illustrates the mechanism of meiosis and the formation of the gametes in this hybrid which possesses the two genes within the same chromosome. The name *linkage* is applied to the coexistence of two or more genes in the same chromosome. Linkage may also be defined as the tendency for two genes to remain together in the same chromosome during hereditary transmission. Linkage of factors was discovered during the analysis of crosses of different varieties of the sweet pea (*Lathyrus odoratus*) which differ in the color of the flowers and in the form of the pollen grains (Bateson, 1905)

In 1910 T. H. Morgan and his collaborators commenced the genetic study of a small fly with grayish body and red eyes, *Drosophila melanogaster* in which a mutation to white eyes appeared. While new crosses were carried out, new phenotypes and mutations were constantly being discovered (flies without wings, without eyes, with short wings, with yellow eyes, without bristles, and so on). From this multitude of new facts, Morgan reached the conclusion that, due to the linkage, all the genes of the fly which are linked are found divided into four groups, and that the genes of each group are linked together. Each group of genes is then transmitted to the progeny as one unit. From this arose the view that the genes of each group are found united in a single chromosome, which serves as their vehicle. In *Drosophila* there are four pairs of chromosomes, each with one group of genes. The first chromosome has about 500 genes, the fourth chromosome, which is the smallest, has somewhat more than ten. Multiple crosses showed that the linkage of the factors is not absolute and that it may be broken with a certain frequency. If a hybrid female  $F_1$  of this insect with the genes "gray" and "long wings" (double dominant) is crossed with a male with the genes "black" and "vestigial wings" (double recessive) we have an unexpected result, since instead of two classes of descendants four are obtained. The first two combinations are the ones expected and appear in 83 per cent of the cases; the other two are new combinations ("gray vestigial wings" and "black, long wings") and appear in 17 per cent of the cases. Calling AB the dominant genes and ab the recessive genes located respectively in each chromosome, the following result is seen



Morgan set forth the hypothesis that the flies composing this 17 per cent are the product of a rupture of the linkage and that the recombination or the formation of new combinations must come about through an interchange of parts between the two chromosomes of the hybrid. This phenomenon was designated

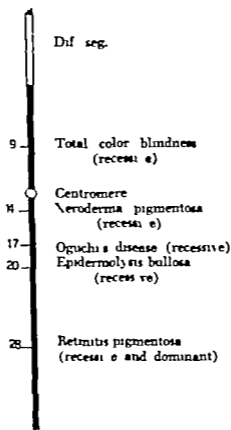


Fig. 95 Genetic map of the X chromosome of man with six genes localized in the region of pairing indicated in black. (After Haldane)

crossing over and this term has become a part of the general literature, being employed in all languages at the present time.

Morgan and his collaborators supposed that the genes have a linear distribution in the chromosomes, that they are located in a constant and definite order and always occupy the same position in the chromosome. Thorough studies have been made of all the classes of combinations of a great number of genes. It has been found convenient to represent the results graphically by the construction of maps of each chromosome showing the topography and respective locations of the genes (Fig. 94). Maize or

corn (*Zea*) which is perhaps the most thoroughly studied of the plants, has been mapped very completely with the localization of more than 400 genes. Chromosome maps also have been constructed for the hen, the mouse, the sweet pea and so on. In the human species a series of genes have been localized in the sex chromosome (Fig 95)

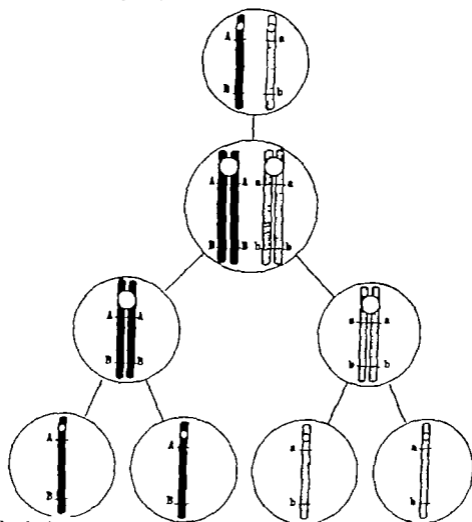


Fig 96 Diagram of the segregation of two pairs of allelomorphous genes localized in one pair of chromosomes (linkage) without crossing over in meiosis. Two classes of gametes AB and ab result.

### Crossing Over and Chiasmata

If two genes are located very close to each other inside the same chromosome the chances are small of crossing over occurring between them and of separation during meiosis. Such genes are apt to stay linked together in which case the four resulting nuclei will have the combination of the parental genes AB and ab (Fig 96)

If a crossing over between the two genes occurs, two of the nuclei will bear parental combinations AB and ab and the other

two nuclei will have new combinations (or recombinations)  $Ab$  and  $aB$  (Fig 97) The farther apart the two genes are in the chromosome, the greater will be the frequency with which a chiasma or crossing over will be produced between them. Consequently the more frequent will be the recombinations in two of the four nuclei from each meiotic process Such data are used in

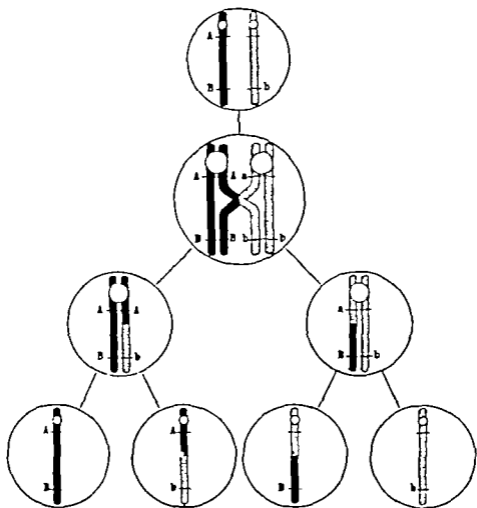


Fig 97 Diagram of the segregation of two pairs of genes located in the same pair of chromosomes in which crossing over has occurred during meiosis (linkage with crossing over) Four different classes of gametes  $AB$ ,  $Ab$ ,  $aB$ ,  $ab$  result.

constructing genetic maps of chromosomes The number of units between two genes on the map is given by the percentage of recombinations among the products of a large number of meioses When two linked genes are found very close together in the chromosome, the percentage of recombinations is low The percentage is equal to half of the average frequency of chiasmata or of interchange between the two genes

Let us give an example If in 100 meioses there are only ten in which a crossing over occurs between two genes, there result

20 recombinations and 20 parental combinations (since ten meioses produce forty nuclei) The other ninety meioses give 360 nuclei ( $90 \times 4$ ) with parental combinations Hence the average of the recombination is  $20/400 = 5\%$  From this one deduces that the distance between the two genes is five units. Considering the case of the cross analyzed on p 221, in which there are 17 per cent recombinations (which signifies that there is a distance of 17 units between the two genes) the frequency of chiasmata is 34 per cent. In the cross of *Drosophila* between "yellow body" and white eyes, there is 13 per cent of recombinations and therefore this corresponds to a frequency of chiasmata of 26 per cent.

According to the theory of chiasmotypy (Janssens, 1909-1924) modified by Darlington (1930) (partial chiasmotypy) "All chiasmata result from crossing over between two chromatids of the partner chromosomes" (Darlington, 1937) The theory holds that at the point of formation of a chiasma there are two chromatids which interchange and two which do not, amounting to 50 per cent crossing over. When in a bivalent there is a chiasma frequency of 10 one infers that in two of its four chromatids an interchange has occurred. In this case, as each crossing over corresponds to one genetic unit, the chromosomes are considered to be 50 genetic units in length. In the same way a bivalent with a chiasma frequency of 35 has 125 units one with 20 has 100 units, one with 40 has 200 genetic units (Table X.)

TABLE X  
FREQUENCY OF CHIASMATA, TOTAL LENGTH OF THE MAP CALCULATED CYTOLOGICALLY AND ACTUAL GENETIC MAP IN MAIZE  
(Taken from White, according to Darlington, 1937)

Chromosomes	Frequency of Chiasmat	Length of the Map in Genetic Units		Number of Genes Present
		Calculated Total	Known Total	
1	3.05	183	102	7
2	3.25	163	88	6
3	3. —	150	92	7
4	2.95	148	80	10
5	2.95	148	44	6
6	2.20	110	52	5
7	2.45	123	50	7
8	2.45	123	20	2
9	2.20	110	32	7
10	1.05	69	68	5
Totals	27.05	1333	618	60

Beadle (1932) studied the frequency of chiasmata and of crossing over in a hybrid of *Zea mays* and found 12 per cent crossing over which corresponds to 24 per cent frequency of chiasmata. This coincides approximately with the frequency actually found (20 per cent)

Since a crossing over determines a chiasma, in diploid and triploid organisms we should have the same number of chiasmata per chromosome. In a triploid individual, we should have  $3/2$  times the number of chiasmata found in a diploid.

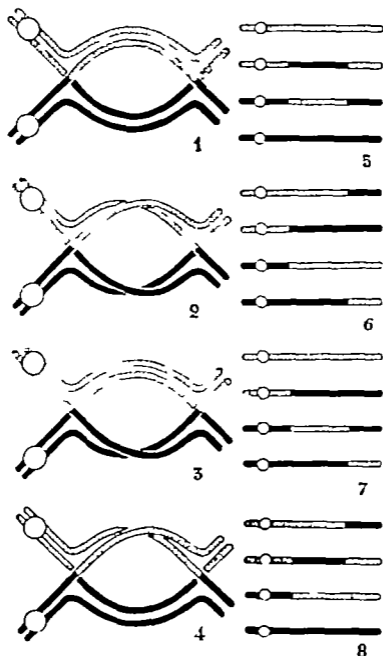


Fig. 98. Different relationships between two successive chiasmata (1, 2, 3, 4) and the products of the double exchange (5, 6, 7, 8) in the same bivalent. 1, 5. Reciprocal pairs of chiasmata (two strands exchange); 6, complementary pairs of chiasmata (four strands exchange); 3, 7 and 4, 8. Diagonal pairs of chiasmata (three strands exchange). (Partially redrawn from White 1915.)

Another manifestation of the relation between chiasmata and crossing over is given by the phenomenon of *interference*, which is the inhibitory influence exerted by one chiasma on the formation of another in a nearby region. If for example the distances between *a* and *b* and between *b* and *c* are small (few units

or less) a crossing over between *a* and *b* prevents another at the same time between *b* and *c*. If a chromosome is short, interference may prevent the formation of more than one chiasma.

**Multiple exchanges** As explained above, when a chiasma is formed there are two crossover chromatids and two non-crossover chromatids (50 per cent of crossing over) In the case of double exchanges (double chiasmata) only two or three or all four strands of a bivalent (2 strand, 3 strand and 4 strand exchange, see Table XI) may be involved (Fig 98) The three types of exchanges occur respectively in the proportion 1 2 1 (see Table XI)

Although it is difficult to distinguish those different types of relationships between the chiasmata cytologically they have been observed in the grasshopper *Melanoplus femurrubrum* (Hearne and Huskins, 1935)

TABLE XI  
PRODUCTS OF DOUBLE EXCHANGES IN THE SAME BIVALENT

Type of Double Exchange Bivalent	Types of Chiasmata Relationship	Types of Chromatids Produced and Their Frequencies		
		Non-crossovers	Single Crossovers	Double Crossovers
2 strand	Reciprocal (1 type)	2		2
3 strand	Diagonal (2 types)	2	1	2
4 strand	Complementary (1 type)		1	

## POLYPLOIDY

In the previous chapter we considered the possibility that organisms may exist which have more than two haploid sets of chromosomes in their somatic complex. In these cases, each chromosome may be represented three, four six or more times. It is said that such an organism is *polyploid* being respectively *triploid*, *tetraploid*, *hexaploid*, and so on The polyploids may constitute a series in which the numbers of chromosomes are exact multiples of the haploid number called *euploids* If the numbers are not exact multiples, they are called *aneuploids* These may be *hyperploids* (example  $5x + 1$ ) or *hypoploids* (example  $6x - 2$ )

Polyploids may originate either by reduplication of the number of chromosomes in a somatic tissue with suppression of cytokinesis, or by formation of gametes with an unreduced number of chromosomes or with more chromosomes than the

normal haploid number. This second type of polyploidy may originate from three causes: (a) irregularity in the first or second meiotic division (lack of pairing, lagging of the chromosomes on the spindle, and so forth); (b) syndiploidy formation of gametes with a double number of chromosomes; (c) suppression of the first meiotic division.

The genetic consequences of these two mechanisms are different. In the former the only difference is quantitative, since the same genes are present as in the original form, although in greater number. However, when nonreduced gametes carry different sets of chromosomes with respect to their allelomorphs, the resulting chromosome complex will be qualitatively and quantitatively different.

There are two types of polyploids: *autopolyploids* and *allopolyploids*. Autopolyploids have chromosomes of the same class; examples are autotetraploids AAAA, autotetraploids AAAAAA, and so on successively (Fig. 99). Allopolyploids originate by the union of two or more sets from different species. For example, a hybrid organism of which the chromosomes may be AA in the diploid, upon reduplication



Fig. 99. Three autopolyploid tomato plants. *a*, Diploid, with 24 chromosomes; *b*, triploid with 36 and *c*, tetraploid with 48 chromosomes. (After Jørgensen, taken from Hurst by Waddington, 1939.)

will form an allotetraploid AAAA of interspecific origin (Fig. 100). As a rule, allopolyploids originate by secondary splitting of a hybrid between species or between genera. When a hybrid allopolyploid has two genomes of each one of the two species which gave rise to it, it is called *amphidiploid* or *didiploid* (example: *Primula leuensis*).

If the doubling of the chromosomes is produced very precociously in the first stages of segmentation of the zygote, the entire individual will be allopolyploid. If it occurs later only some parts of its body will be allopolyploid. *Primula leuensis* ( $2n = 36$ ) is a case of an allopolyploid, since it originated from a cross between *P. floribunda*  $\times$  *P. verticillata* ( $2n = 18$ ). The first descendants of this cross have 18 chromosomes, but soon, by duplication of one set, a line with 36 chromosomes originates. This case may be represented thus:  $(9 + 9) \times 2 = 36$ . Allopolyploid organisms are also formed by the crossing of individuals with different numbers of chromosomes, such as occurred in the well known case of *Nicotiana glauca* ( $2n = 6x = 72$ ), a synthetic species formed by *N. glutinosa* ( $2n = 2x = 24$ ) crossed

with *N. tabacum* ( $2n = 4x = 48$ ) (Clausen and Goodspeed) An allohexaploid with two genomes of *glutinosa* and four genomes of *tabacum* was formed. The general formula in cases such as the example just given is  $(x + 2x) \times 2 = 6x$ .

In a similar manner allotetraploids may originate from a cross of a diploid species (with nonreduced gametes) with a tetraploid species. Some species in nature have been formed in this way. The Argentine sorghum, *Sorghum alnum* Parodi ( $2n = 4x = 40$ )

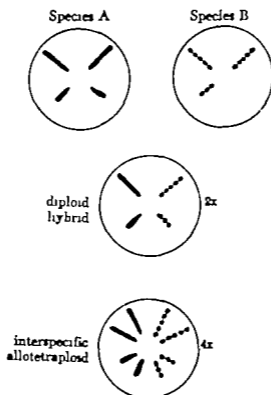


Fig. 100 The formation of an interspecific allotetraploid.

which is an allotetraploid may have originated by a cross between *Sorghum alepense* ( $2n = 4x = 40$ ) with a diploid sorghum ( $2n = 2x = 20$ ) (Saez and Nuñez, 1943).

Knowledge of the meiosis of polyploids is of importance in determining the origin of the individuals which have taken part in the formation of species. Thus, study of the affinities and differences which are manifested during the zygotene pairing and the formation of the chiasmata in the diplotene stage may furnish keys to the probable phylogeny of a species, depending upon whether the organism is autopolyploid or allopolyploid. In an autopolyploid species there are three or more chromosomes which come together to form multivalent chromosomes, so that the individual may be triploid, tetraploid, pentaploid, hexaploid,

and so on. In the pairing of any polyploid *two chromosome filaments (and never more than two) are always paired in a particular segment whatever may be the number of homologues and whatever may be the place of contact during pairing* (Fig 101) However configurations which appear may be extremely varied.

In the case of a meiosis of an autotetraploid individual with forty chromosomes the formation of ten tetravalent chromosomes is seldom observed, since the competition between four equal

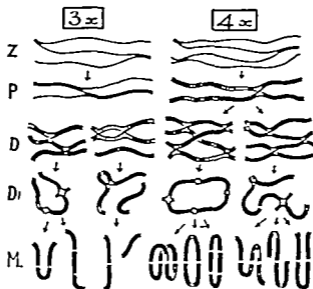


Fig. 101 Diagram of the meiosis of a triploid ( $3x$ ) and a tetraploid ( $4x$ ). Z, zygotene P pachytene D diplotene D<sub>1</sub>, diakinesis M metaphase I. The behavior is subject to chance during the zygotene in the pairing, during the diplotene in the formation of the chiasmata and during the metaphase in the orientation of the spindle. (After Darlington, 1937)

chromosomes diminishes the possibilities for the formation of chiasmata. Such formation depends on the length of the chromosome and on the time for pairing (greater or less rapidly for conjugation in the zygotene stage). The long chromosomes delay more than the short ones hence more univalents are formed in the former case than in the latter. If chiasmata are formed, the chromosomes remain united. If chiasmata are not formed, univalent elements are always found in an erratic position at metaphase being distributed irregularly during the anaphase to one or the other pole. The incomplete pairing which is seen in the autopolyploids led Darlington to set forth the theory that the association of homologues depends on the formation of chiasmata. This incomplete association is the reason why the segregation of the homologous chromosomes may be very irregular during the

anaphase, bringing about the formation of gametes having incomplete sets of chromosomes

The arrangement and orientation of the multivalents on the spindle is also very irregular. For example, instead of a tetravalent being formed, one may find one trivalent + one univalent, one bivalent + one bivalent, or one bivalent + two univalents. The segregation of a tetravalent in the form of a ring produces the separation of two centromeres to one pole and two to the other. On the other hand, if the tetravalent is in the form of a chain, the separation will be  $2 + 2$ ,  $3 + 1$ , or the four centromeres at the same pole. The gametes formed in such cases will not be functional and therefore the individual will be sterile. This is the cause of the sterility which is often seen among the autotetraploids.

Among the true allotetraploids, the pairing is more regular than in the autotetraploids and at times is as normal as among the diploids. Thus, an individual with four chromosomes,  $AAA'A$ , during the meiotic divisions, forms pairs of bivalents  $AA$  and  $A'A$  which leads to the formation of well balanced and fertile gametes. An allopolyploid, because of its meiotic regularity is more fertile than an autopolyploid. Thus fertility depends also on the similarity among the individuals, species or genera which come together to form the chromosome group of the allopolyploid.

There are two types of pairing in the meiosis of the allopolyploids, *autsyndesis* and *allosyndesis*. For instance, let us suppose we have a case of an allotetraploid which has two sets of chromosomes from one individual and two sets from another. If in one set,  $AAA'A$ ,  $A$  and  $A$  are homologues and  $A$  and  $A'$  also are homologues, and the bivalents are  $AA$  and  $A'A'$  we then have autsyndesis (a homogenetic association). But if on the other hand,  $A$  is paired with  $A'$ , bivalents of distinct or heterogenetic origin will be formed. In this case we have allosyndesis because phylogenetically different chromosomes are united.

Analysis of meiosis in allotetraploid species is difficult when one must discriminate between the origin and the forms of pairing of the chromosomic sets which have come together to produce them.

In some species, particularly in those which have small chromosomes, the bivalents are seen in groups very close together (although without material connections) in the first as well as in the second meiotic division. The term applied to this type of union is *association* or *secondary pairing*. This has much importance in the investigation of the possible affinities (homologies) of the chromosomes of an allopolyploid.

Secondary polyploid are organisms in which some of the chromosomes of the diploid complex have been reduplicated. Thus in the apple, instead of 14 chromosomes which is the typical number ( $x = 7$ ) one finds individuals with 17 chromosomes ( $2x + 3$ ). In this case the haploid set of 7 chromosomes (from  $A$  through  $G$ ) is represented in the proportions  $AAA$ ,  $BBB$ ,  $CCC$ ,  $DD$ ,  $EE$ ,  $FF$ ,  $GG$  which have originated through an unequal reduplication. It is possible that secondary

associations are a consequence of secondary polyploidy. In general, in the autopolyploids, segregation and the formation of the gametes occur in an irregular manner.

### Haploids

Some exceptional plants and animals have a monoploid set of chromosomes. The individuals are small and generally sterile. They have very irregular meiosis because of the absence of homologous chromosomes which could pair together. In these individuals there are only univalents which give rise to gametes with a varying number of chromosomes. In a haploid of maize, one may find a gamete with ten chromosomes, one for each 1024 divisions. The haploids have, nevertheless, considerable usefulness for obtaining homozygotic types in the event of fortunate autofecundation.

### Polysomy

When one or more of the chromosomes are reduplicated and are found represented three or more times the organism is said to be polysomic. Thus is a special kind of aneuploidy owing to a faulty separation of chromosomes in the meiotic process. One of the chromosomes, together with its homologue, passes to the same pole and, after the completion of division II, proceeds to form a part of the same gamete. Such a phenomenon is designated as *nondisjunction*. Such a gamete, upon union with any normal gamete will give rise to an individual called *trisomic* ( $2x+1$ ) in which, during meiosis, the three chromosomes are joined together to form a trivalent element.

Among the trisomic organisms are some races of *Drosophila*. But the more common examples are among the plants such as in the maize, *Crepis*, *Matthiola*, *Solanum*, and especially in *Datura* (Blakeslee, Belling and collaborators). In the last plant the presence or absence of a definite somatic characteristic is seen as a function of the presence or absence of a definite chromosome. The experiments of Blakeslee on *Datura* demonstrate the influence of genic equilibrium on the expression of the characteristics of an individual and lend strong support to the concept of each chromosome functioning as a morphogenetic factor.

There also are double trisomics ( $2x+1+1$ ) and double tetrasomics ( $2x+2$ ) as well as secondary and tertiary pentasomics, which are formed by the translocation of chromosomes of the same class. Genetically a trisomic, by having triplicated a particular chromosome, shows a progeny in the proportion of 35 : 1 (instead of 3 : 1).

Those individuals which have in their cells  $2x-1$  chromosomes are called *monosomics*. This condition occurs when an irregular gamete ( $x-1$ ) joins with a normal gamete, producing a zygote ( $2x-1$ ). In general, monosomic individuals are neither vigorous nor very fertile, but this is not always the case. In meiosis, the solitary chromosome has no partner and is in an erratic position on the metaphase plate. McClintock found that in maize these chromosomes often pair within themselves by curving and if by chance a chiasma is formed, the result is an inversion. In *Nicotiana*, *Datura*, and so forth, and among animals, there are cases of the monosomic condition. From the genetic point of view monosomic organisms are interesting because they have genes without mates (lacking a homologue). This fact allows one to follow the distribution of the recessive genes located in the unpaired element and to determine the values of linkage and of crossing over in the progeny.

*The scarcity of polyploids among animals is due to the bisexuality which characterizes the majority of animal species. This implies a special mechanism in which sex is determined by the segregation of a pair of differentiated sex chromosomes X and Y or of an unpaired X chromosome.*

The fact that one of the two sexes has two different types of gametes, X and Y or X and O (O signifies the absence of the other sex chromosome) creates a difficulty which may lead to sterility or to the production of sexual anomalies. As will be seen later in this chapter normal sexuality depends on the genetic equilibrium existing between the sex chromosomes and the common or somatic chromosomes (*autosomes*). If this equilibrium is broken, sex aberrations occur, and individuals of this type cannot carry out reproduction. If polyploidy occurs, the mechanism is disturbed and the race or species may disappear because of sterility. In the case of hermaphroditic or parthenogenetic animals like *Artemia salina* and *Trichoniscus provisorius* (Crustacea), polyploid individuals are frequently encountered. The parthenogenetic races of these species cannot cross, since they are polyploid, while the diploid races reproduce sexually in a normal way. In *Artemia* the sexual race has 42 chromosomes and the parthenogenetic races 84 and 168 (tetraploid and octoploid). In *Trichoniscus* the individuals of the diploid races are bisexual with 16 and the triploid are parthenogenetic with 24 chromosomes. Among the insects (Lepidoptera and Coleoptera) polyploidy likewise is found related to parthenogenesis. The butterfly *Solenobia* presents forms with 62 (diploid) and 124 (tetraploid) chromosomes, the latter being parthenogenetic (Seiler). The occurrence of polyploid individuals is very limited but the presence of cells with their chromosome complexes reduplicated is frequently observed among animals. They are often seen in spermatocytes brought about by faults in the spermatogonial divisions. This phenomenon is useful to a biologist since it permits one to follow more easily the behavior of the chromosomes.

### Experimental Production of Polyploids

At present it is possible to induce the reduplication of the chromosomes of an organism by the use of substances such as colchicine, acenaphthene, heteroauxin and veratrine, and also by subjection to heat or cold. The method most frequently employed is the alkaloid, colchicine, which is used in 0.5 to 2 per cent solutions, seeds being immersed in it at the beginning of germination. Injections of colchicine into the young plant have been attempted with good results. The substance acts efficiently in producing the development of a polyploid plant. Perak (1943) obtained tetraploid plants of maize and of *Triticum durum* with this technique. Tetraploids also have been produced in *Sorghum sudanense* (Salomón) in *Petunia* (Perak), *Gaillardia pulchella* (Schnack) and in the soy bean (Andrés).

The action of the chemical substances is exerted by inhibition of the formation of the spindle, once the chromatids have separated, and, in this way cytokinesis is not completed. When some time has passed, the cells regain their normal activity beginning again their accustomed rhythm, but with a double number of chromosomes. The possibilities offered by the experimental production of polyploids, both from the standpoint of scientific and applied work, are innumerable.

## THE ALTERATION OF THE CHROMOSOMES AND THE MECHANISM OF THEIR REORGANIZATION

The harmony of the genetic system is evidenced by the constancy of the arrangement of the hereditary material carried in the chromosomes. Nevertheless, changes may occur in the chromosomes, brought about by accidents which disturb their regularity and produce disarrangements in the structure of their parts.

Although such changes are sometimes found in nature, most of the knowledge of these changes has been possible due to the employment of experimental methods which increase their frequency. In organisms subjected to the action of  $\alpha$  rays,  $\gamma$  rays, ultraviolet rays, to the influence of chemical substances, or to rapid changes in temperature or in centrifugal force, the frequency of the alterations is increased, thus providing a valuable means for the analysis of the genetic and structural organization of the chromosome in relation to the mechanism of mutation and the evolution of living organisms.

The changes in the chromosomes can be summarized in the following classification:

- 1 Genic mutations (intragenic)
- 2 Numerical alterations of the chromosomes
  - a Diminution or increase of the number of complexes *haploidy* or *polyploidy*
  - b Increase or diminution of one or more chromosomes of a complex *polysomy*
- 3 Structural alterations in the segments of the chromosomes
  - a Intrachromosomal rearrangements (intergenic) *inversions*
  - b Interchromosomal rearrangements *translocations*
  - c Loss or increase of chromosomal segments *deficiencies and duplications*

We shall consider here the processes comprised under the third point. The delicacy of the chromonema in certain stages of the interphase and prophase facilitates the production of various alterations, since in many instances the filaments come in contact. This makes possible the breaking and rejoining of the filaments, giving rise to a different organization of the parts. In general, the name *structural hybrids* is assigned to organisms which present such changes.

When a chromosome is fractured, the pieces may fuse in the same form in which they were originally or recombine in a

different manner. In the latter case, the new arrangement becomes 'visible'. When the rupture is in the same chromosome, it is called *homosomal* and when it occurs in different chromosomes, *heterosomal*. If the fracture is located in the same arm of the chromosome, the rearrangement is *paracentric*, whereas if it occurs in different arms, on both sides of the centromere, it is *pericentric*.

In order to understand the mechanism of the reorganization of the segments of the chromosome, we must first refer to the theories which have been proposed to explain them. In true crossing over the segments are transposed by a breakage followed by a refusion of the chromatids, but without changes in the arrangement of the genes. However, if the segments which are transferred are of different size, it is said an *illegitimate crossing over* has occurred. According to Belling structural changes have originated from such abnormal crossing over. This phenomenon may be interpreted according to the theory proposed by Stadler of "*previous fracture and later contact*," that is, the independent fracture of two chromosomes with their later union. The fracture probably is produced by the destruction of one or more genes or of the intergenic connections. Experiments with x rays tend to confirm this interpretation.

Translocation is transposition of two segments between nonhomologous chromosomes. In general, translocations are produced by rupture of two nonhomologous chromosomes followed by a transference and fusion of the reciprocal segments. For this reason, it is called *reciprocal translocation* or *segmental interchange*. The result may be two chromosomes, each one with a centromere (Fig. 102, c) or one chromosome with two centromeres (*dicentric*) in addition to one segment (or fragment) without a centromere (*acentric*) (Fig. 102, d). In this last case these chromosomes are lost in the first cell divisions because of mechanical irregularities.

The rupture may occur in any section along the length of the chromosomes, elements of varying length resulting (Fig. 102, b). Translocation may be homozygotic or heterozygotic. In the latter case, the individual has two complete chromo-

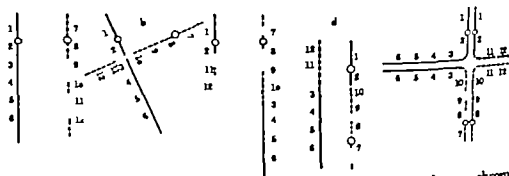


Fig. 102. Process by which a translocation between nonhomologous chromosomes occurs. The two possible results are shown at c and d. In e the configuration which originates on pairing the reorganized chromosome with the other normal homologue during the pachytene stage is shown.

somes with the genes arranged as before, and two translocated chromosomes which have a different arrangement of their genes. The individual is called a *translocation heterozygote*. In a translocation heterozygote, of the formula

$$\frac{AB\ CD\ \quad\ EFGHIJK}{AB\ FE\ \quad\ DC\ CHIJK}$$

the chromosomes at the time of pairing constitute a bivalent in the form of a cross (Fig. 104, c) which, at the end of this stage (by repulsion of the chromatids) opens and forms an annular element. The segregation of the four chromosomes gives six classes of gametes.

An interesting result occurs when the translocation is very close to the centromere and there originates a V-shaped chromosome with two arms in the form of V and a small fragment which tends to be eliminated because it is practically inactive, even though it has a centromere (Fig. 103, A). Fusion of this type is called

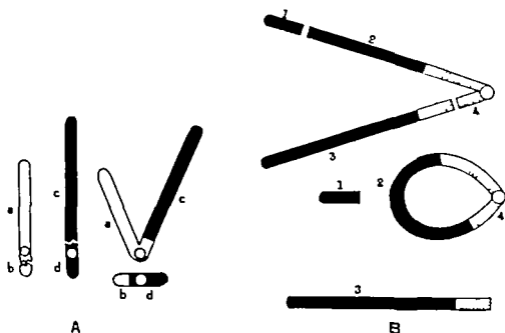


Fig. 103. A the origin of a new chromosome in the form of a V by fusion of two nonhomologous chromosomes after reciprocal translocation; the piece *bd* is lost. B formation of an annular chromosome in *Drosophila* derived from two V chromosomes fused to form a V; the fragments 1 and 3 are lost.

*centric fusion*. In this way a new chromosome is constituted and, at the same time the somatic number of the species is decreased. In many animal and plant groups a chromosomal mutation seems to have been produced in this way by diminishing the number of chromosomes and giving rise to a new type of chromosome and thus to a new race or species.

In this manner there were formed in two species of the genus *Aleuas*, two V chromosomes (metacentric) which reduced the diploid number from 23 to 19 (Fig. 104). The species *A. vitticollis* and *A. lineatus* have been maintained in nature with this alteration, perhaps for a long time. In the latter species translocation occurred between two nonhomologous chromosomes, as may be seen by the difference in the arms of the V (Suzuki, 1931, 1935). There is a cultivated race of *Drosophila* which has two V chromosomes joined together by a centromere thus constituting a V. If a fracture is produced between the arms of the V in two regions of this element ("fused V") an annular chromosome (closed V) is formed (Fig. 103,

B) This alteration produces, furthermore, a duplication and a deficiency which does not affect the viability of the organism, but does cause a considerable degree of sterility



Fig. 104 Process by which four chromosomes in the form of a V originate in the genus of locusts *Aleuas*, in which the number of chromosomes has been reduced from 23 to 19 by reciprocal translocation between nonhomologous chromosomes. In the upper row the actual composition of the chromosome complex of the genus is shown. In the lower row the chromosomes are shown as they probably were before the production of this alteration. (After Saenz, 1931)

**Inversion (Fig. 105, A)** If the central segment of a chromosome which has broken into three parts is inverted and becomes fused in this reversed position, having effected a rotation of 180 degrees, we say that an inversion has occurred. As was said on page 164 the external segments do not tend to fuse in an inverted manner after breakage, because of the peculiar property of the telomeres.

When an inversion occurs in one of the chromosomes of a pair of homologues, the individual is designated an "inversion heterozygote." In order to conjugate, the normal chromosome must then make a turn in order to adapt itself to the inverted homologous chromosome (Fig. 105 a). If the crossing over is carried out

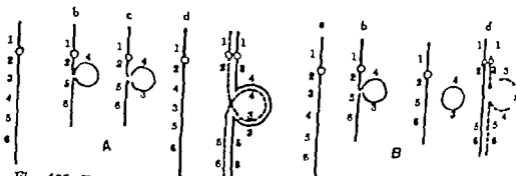


Fig. 105 Diagram to illustrate the production of an inversion at A and of a deficiency at B and the method of pairing in the pachytene stage in the two cases (e and d respectively). The fragment 3-4 of B is lost during cell division. At e the normal homologous chromosome must form a curve during its pairing with the inverted chromosome represented by the continuous line. At d, the normal chromosome (dotted line) also is adapting itself for pairing with the altered chromosome.

between a pair of heterozygotic homologous chromosomes, an inversion may occasion important consequences, particularly if the interchange produces a chiasma within the inverted segment (Fig. 106, B). Under these circumstances after the interchange between the chromatids has been effected, there results at the anaphase what is called a dicentric chromatid bridge. This bridge consists of one chromatid with two

Recently Catchende and Lea (1945) using x rays, have found that the fractured ends of the X chromosomes may unite in some cases so as to produce terminal inversions in *Drosophila*.

centromeres. The bridge is drawn out until it ruptures when the chromosomes separate toward the poles. At this same time, an *acentric* chromosome (without a centromere) forms, which is a fragment resulting from the rupture (Fig 106, B). The case which we have described is frequently seen and is a cytological index of considerable importance in localizing an alteration of this type.

Prior to the discovery of this cytological index, inversions were identified genetically in *Drosophila* by proving the absence of crossing over. When, in the course of such genetical analysis, an unexpected diminution of the frequency of recombinations was noted, an unknown factor called "the suppressor of crossing over" was postulated. At the present time it is known that this phenomenon is due to the crossing over in an inverted segment.

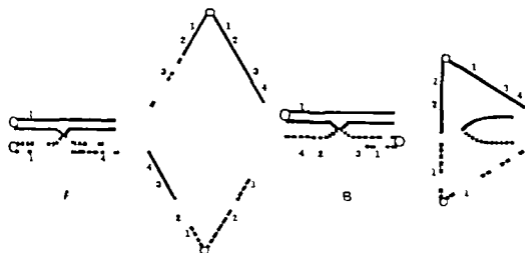


Fig 106. Diagram to show the occurrence at A of a normal crossing over between two chromatids and the result of this at the time of the anaphase at B the crossing over between two chromatids, one normal and the other inverted (4 2 3 1). The result of this crossing over is seen in the anaphase with the production of a fragment (3 4 2 4) without a centromere and an anaphasic (dicentric) bridge (1 2 3 1) with two centromeres. The fragment is lost.

Inversions are called *paracentric* if the centromere is situated in the arm of the chromosome. They are *metacentric* if the centromere is within the loop. When a chiasma takes place within a paracentric inversion, the result will be two chromatids, one acentric and the other dicentric (Fig 106, B).

If two complementary chiasmata are formed at the limits of one inversion, two acentric and two dicentric chromatids will be formed. The last two form a double bridge at the anaphase. When chiasmata are reciprocal, the inversion produces chromatids without modifications. Where the two chiasmata are diagonal inside the inversion, metaphasic chromatids similar to those shown in Figure 106 B are formed.

Loop chromatids may be formed at the first anaphase if one of the chiasmata is situated between the centromere and the inverted segment and the other inside the inversion. These loop chromatids have both ends attached to the two halves of one centromere. In this case at the second anaphase a dicentric bridge forms (Fig 107).

Inversion plays an active role in the evolution of the species, since they change the arrangement of the genes in the chromosome producing structural mutations in the individual. It is possible to determine numerous inversions in hybrid species which give clues to their phylogenetic origin. At the same time they play an important role in the mechanism of isolation among species, which consists of reciprocal arrangement and changes in the number and relative positions of the chromosomes.

Of great significance are the investigations carried out by D. B. D. and collaborators on the polytenic chromosomes of various species of *Drosophila*.

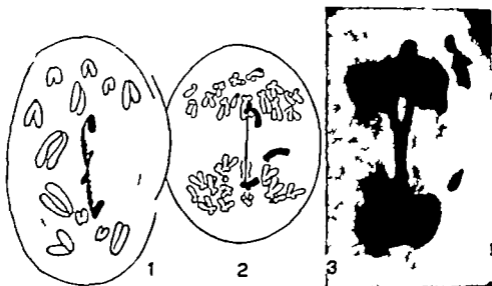


Fig. 107 Three cases of dicentric bridges produced. at 1 by crossing over between chromosomes with inverted segments. The fragment is very small because of the shortness of the inverted segment, at 2 the fragment is large because the inverted segment is long at 3 there are two chromatin bridges produced by a translocation of somatic chromosomes brought about by the action of centrifugal force. (1 *Schistocerca gregaria*, after Saez, 1945 2 tulip, after Upcot, 1939 3 sweetpea (*Lathyrus*) after Saez, 1941)

The evolution of the chromosomes of the genus *Drosophila* constitutes a special chapter in cytogenetics which is too extensive to analyze here. By comparison of the chromosomes of different individuals it was seen that distinct arrangements exist among the genes. Seventeen different ways of overlapping inversions were identified within the segments already inverted. It was demonstrated further that there are,

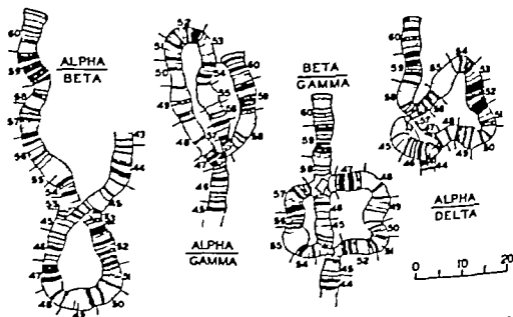


Fig. 108. Inversions in the polytene chromosomes of *Drosophila dentissima*. The Beta type has the segments from 45 to 53 inverted and the Gamma type has an inversion from 46 to 57. The pairing of the Beta and Gamma types gives rise to superimposed inversions in the form of an 8. (After Dobzhansky and Sokoloff, 1939)

in different geographical localities, distinct racial groups based on changes of the chromosome III. The species of *Drosophila* differ among themselves according to the different arrangement of their genes which have resulted from successive rearrangements of the chromosomes during the course of their phylogeny (Fig. 103). Among plants, inversions frequently occur and, as a result of the pairing of structurally unlike chromosomes, gametes with new types of chromosomes may arise. An interesting phenomenon appears in the progeny of the cross of *Licia satira* with *L. amphicarpa*, where the new chromosomes are lethal if homozygous, but are viable if they are in a heterozygous condition (Sveshnikova, 1936).

Duplications and deletions usually originate as a consequence of a reciprocal translocation which increases or diminishes the length of the chromosome and the number of genes (Fig. 105 B). When a chromosome breaks into three parts and loses the middle segment, a *deletion* or loss has occurred. When the segment exceeds a certain length, the effect is lethal. In *Drosophila* many deletions are known, such as yellow body and "notched," which is a mutation of the wings. This latter is found in chromosome I (X) and is produced by the loss of a segment of 1.5 units. It is lethal in the males and, when homozygous, also in the females. Thus the flies which continue living with this deficiency are heterozygous.

*Repeat* is the term applied when a chromosome alteration is due to the repeated duplication of a small segment of a chromosome producing, for example abcdababcd. This is produced by crossing over between unlike segments. It occurs in Bar the narrow eyes in *Drosophila*, a mutation formed by repetition of one zone of chromosome I which, when repeated again, gives rise to the variety "Ultrabar" or "Double bar."

### Position Effect

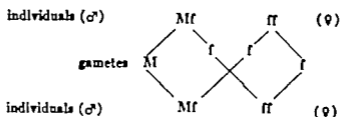
In general, inversions and translocations do not disturb the development of the individuals. However sometimes phenotypic changes occur. Such changes are due to the position effect. Translocated genes sometimes show changed effects, perhaps due to the influence of adjacent genes. This brings about a modification in the development of the individual. If the gene is returned again to its ordinary location, normality is re established.

## SEX DETERMINATION

Since the beginning of Mendelism, it has been thought that the origin and determination of sex was intimately related to heredity. The commonly observed fact that individuals of male and female sex are found in nature in more or less equal proportions was the point of departure for the genetic study of sex determination. Cytology and genetics have given the key to the mechanism which governs this process. Genetic investigation demonstrated that maleness or femaleness is generally transmitted from one generation to the next in the same way as any other hereditary characteristic. One of the individuals behaves as though heterozygous in relation to a genetic factor determining sex. This individual (since it is a hybrid in this respect) forms two classes of gametes while the other (homozygous) produces a single class of gametes. Therefore only two types of fertilization are possible resulting in 50 per cent males and 50 per cent females.

Correns (1907) working with a plant (*Bryonia*) was the first to demonstrate that sex may be determined according to the laws

of Mendelian segregation. The male of this plant is heterozygous for sex (since it has two classes of pollen grains) and the female is a homozygous recessive. This case behaves as if one were dealing with a cross of a hybrid with a double recessive



If femininity and masculinity are genetic characters, there must be sexual genes in the chromosomes and therefore qualitative and quantitative differences between the sexes. The characteristics

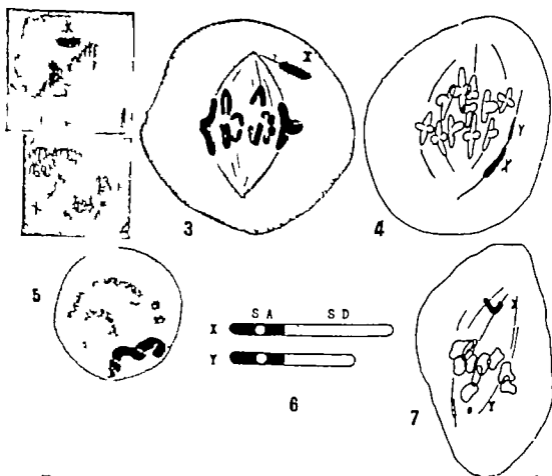


Fig. 109 Sex chromosomes. 1 2 3 the unpaired X chromosome passing toward the poles during metaphase (1 and 3) and anaphase (2); 4 the X and Y chromosomes of the red weasel during metaphase I; 5 the spirialized sex chromosomes in positive heteropyknosis during meiotic prophase in the locust *Dichroplus arrogans*; 6 diagram of the X and Y chromosomes showing the pairing segment (SA) and the differential segment (SD) 7 the chromosomes X and Y of the human species in anaphase, with the autosomes in metaphase. (After Saez, 1930, 1938 and 1945)

of the chromosomes usually furnish the basis for the determination of sex, which takes place at the time of fertilization.

In the majority of diploid bisexual organisms there is a pair of chromosomes which, in the course of evolution, has been specialized for sex determination. In one of the sexes there exists a pair of *sex chromosomes* (also called the  $\chi$  chromosomes, heterochromosomes, and so on) and in the opposite sex a single  $\chi$  chromosome, which is unpaired or paired with a  $\chi$  chromosome (Figs. 109 and 110 B). Thus one sex is homogametic ( $XX$ )

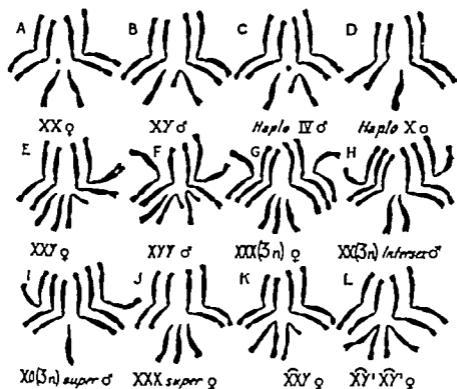


Fig. 110 The chromosomes of *Drosophila melanogaster*. A B normal female and male. C, D E, F G H I J K L, different types of alterations. C D animals with  $2n \text{ IV}$  and  $2n \text{ X}$  respectively. K L, individuals with  $2n \text{ X}$  fused as a  $\chi$  and with  $\chi \chi$  fused, respectively. For the other cases, see the text. (After Darlington, 1937.)

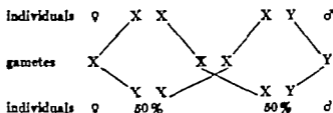
and the other sex heterogametic [ $(XY)$  or  $(XO)$ ]. In many organisms the male is heterogametic and the female homogametic, producing respectively two classes of spermatozoa or spores and one class of ova. But in the insects of the orders Trichoptera and Lepidoptera, certain fishes, a urodele amphibian (axolotl), certain reptiles, and birds the female is heterogametic and the male homogametic. In such organisms the females form two classes of gametes and the males a single one.

The history of the discovery of the sex chromosomes goes back to the time of Henking who, in 1891, noted the presence of "a peculiar chromatic element" in the spermatogenesis of *Pyrrhocoris*. This element he confused with a nucleolus.

McClung (1891) identified this element, giving to it the name of "the accessory chromosome." (Fig. 109 1 2 and 3) We owe to the same investigator the original idea of attributing to the accessory chromosome the function of sex determination (1901 1902) Wilson and Stevens, in a series of important investigations, supported the hypothesis of McClung, establishing for all animals the chromosome mechanism of sex determination. In 1923 sex chromosomes of the type XY and XX were also found in plants.

The human is characterized by the diploid number of 48 and the sexual type XY (Painter 1921, 1923) In man the X chromosomes are larger than the Y chromosomes (Figs 83 15, and 109 7), this is also true in the majority of organisms (Fig. 109 4, 7)

The sex chromosome mechanism should result in equal proportions of the two sexes



Meiosis is normal in the XX pair but in the chromosomes XY there is, at times, a small region with homologous genes. Pairing and crossing over is limited to this region (see below) The sex chromosome is composed of two regions the *pairing segment* and the *differential segment* The homologous region corresponds to the pairing segment and the differential region is that which influences sex determination (Fig. 109, 6)

The most primitive mechanism for sex determination involves the XY type. From this there evolved gradually the XO. The chromosome Y was slowly shortening down to the point of disappearance, since this element is almost entirely inert and has only a few active genes. Later we shall see that this chromosome does not participate actively in sex determination. The more primitive the X and Y chromosomes are, the smaller the differential region and the longer the homologous region. The opposite is found in higher organisms.

Among fishes the XY condition is found initially and there is a high degree of mutability as a result of frequent crossing over between the two chromosomes. The process of involution of the Y chromosome (considered as an X in regression) is concomitant with the frequency of mutations. Thus fishes show the first steps of the chromosomal mechanism of sex determination. As the pairing segment is reduced in size, the possibility of crossing over becomes less and finally disappears. This facilitates the elimination of the Y chromosome from the chromosome group, with out grave consequences for the species. This has occurred in the Orthoptera, Coleoptera, Hemiptera, some Diptera and in the nematodes. In hermaphrodites, since there is an equilibrium between the genes of the male and female conditions, sex chromosomes do not exist or if they do, are not differentiated cytologically. Amphibia also show early steps in the evolution of the mechanism of the sex chromosomes, which appear to have originated from a pair of ordinary chromosomes (autosomes) which later became specialized for the function of sex determination (Witschi, 1934; Saez, 1937)

## The Equilibrium of the Genes and Sex Determination

A series of experiments by Bridges (1913 1914 1916) demonstrated that in *Drosophila* sex chromosomes are not always segregated regularly since for each 2500 meioses there is an ovum which contains XX instead of an X. This *nondisjunction* of the X chromosome produces ova with two of these elements (XX) which, upon fertilization by spermatozoa (Y) give rise to *exceptional females* (XXY) (Fig 110 E) Such a female (white eyes, recessive), crossed with a normal male (red eyes) gives the following progeny

X	Ova	Spermatozoa	Zygotes	Individual	Frequency
1	X	Y	XY	Female red eyes	23
2	XX	Y	XXY	Generally dies	2
3	XX	Y	XXY	Female red eyes	23
4	X	X	XX	Male red eyes	2
5	X	X	XX	Male white eyes	23
6	XX	X	XXX	Exceptional female white eyes	2
7	XY	X	XXY	Male white eyes	23
8	Y	X	XY	Dies	2

This classical cross was, at the time, one of the strongest proofs in favor of the chromosome theory of heredity in that it gave evidence that genes linked to sex (different from the sex genes) occur in sex chromosomes

This experiment also shows that if an ovum having two X chromosomes is fertilized by a spermatozoon carrying a Y chromosome the result is a female instead of a male (Case 6 in the table) If an ovum lacking the X chromosome is fertilized by a spermatozoon carrying the X chromosome a sterile male (with nonmotile spermatozoa) is formed

If we designate the set of autosomes by A, a female of *Drosophila* will have the formula XXAA, and the male XYAA, with their respective gametes (XA) (XA) and (XA) (YA) But, since the Y chromosome is inactive, the Y may be omitted and the male considered XAA If one supposes the factors of femaleness (F) to be lodged in the X chromosomes since the Y is inert the factors of maleness (M) would be located in the autosomes This led to the concept that sex depends upon various genes which must occur distributed among the autosomes and the sex chromosomes. Thus it appears that this is a case of genic equilibrium which determines the sex of an individual

X(F)X(F)A(M)A(M) female      X(F)A(M)A(M) Male  
                                  FFMM                                   FMM

whence

FF > MM > F

Sex seems to depend on the quantitative relation between the number of X chromosomes and that of the autosomes, or between the factors F and M (Goldschmidt 1915)

### The Disturbance of Genic Equilibrium and the Origin of Sexual Aberrations

The problem of sex determination is further complicated by the finding of individuals with an intermediate sexuality and of supersexed and hypersexed individuals. In *Drosophila*, a triploid female crossed with a normal male gives rise to four classes of ova, which, when fertilized by the two classes of spermatozoa from a normal male, produce the following progeny (Bridges 1922, 1925, 1932)

Ova	Spermatozoa	Zygotes	Individuals	Relation $\frac{X}{A}$	Figure 110
2X2A	XA	3X3A	Triploid female	1.0	G
1X1A	XA	2X2A	Diploid female	1.0	A
2X1A	XA	3X2A	Super female	1.5	J
1X2A	XA	2X3A	Intersex	0.67	H
2X2A	A	2X3A	Intersex	0.67	H
1X1A	A	1X2A	Male	0.5	B
2X1A	A	2X2A	Diploid female	1.0	A
1X2A	A	1X3A	Super male	0.33	I

In a normal female the relation  $X/A$  is 1.0 because each X is equivalent to a set of autosomes A, whether the animal is triploid or tetraploid. In the male, the relation is  $X/2A$  or  $1/2 = 0.5$ . In the intersexual female the relation is  $2X/3A$ , that is  $2/3 = 0.67$  which is a relation intermediate between the normal male and female. Therefore, sexuality is conditioned not by the absolute number of the X chromosomes, but by the ratio between the number of X chromosomes and that of the autosomes.

If the proportion of X chromosomes rises so that  $X/A$  equals 1.5 the individual is a super female. But if the number of X chromosomes is less than half that of the autosomes ( $X/A = 0.33$ ) the individuals are super males. In spite of the fact that these organisms are supersexed, they are incapable of reproduction, just as is the case with the intersexes. There is, then, a particular grade of sexuality which permits reproduction.

The concept of genic equilibrium explains why an individual

2X2A is a female. This is because the feminizing tendency of the X chromatin is more powerful than the masculinizing tendency of one set of autosomes. The genes F are stronger than the genes M. Bridges introduced the notion of the *sexual index* representing the female tendency X by 100 and the masculine A by a somewhat smaller number, 80.

Later studies by Dobzhansky and Bridges on the development of the intersexes showed that there is a *turning point* which inclines the individual toward one or the other sex. For example, the intersexes begin their development as males and later turn toward the feminine side. The characters which are determined before the turning point are masculine and those which are determined afterwards are feminine. The final result or grade of intersexuality depends on the time and the state of development when the change is produced.

#### Monogenic or Plurigenic Nature of the Sex Factors

In order to know whether the factor of femaleness F is localized at a fixed point of the X chromosome or is the result of the action of various genes  $F_1, F_2, F_3$  localized at different points on the chromosome, Dobzhansky and Schultz (1931-1934) using breakages produced by x rays, introduced fragments of X chromosome into an intersex 2X3A (the factors of which are FFMMM). They demonstrated that the longer the fragment introduced, the greater is the feminizing influence on the intersexes. Therefore, probably not a single gene but several genes are acting, and these are found distributed along the length of the chromosome. With regard to the autosomes, chromosomes II and III seem to be determiners of the masculine sex and chromosome IV seems to have little influence. Sex in *Drosophila* is probably determined by the equilibrium between two systems of polygenes in the X and in the large autosomes (White, 1945).

#### Disturbance of Genic Equilibrium without Modification In the Number of Chromosomes

Using the butterfly *Lymantria dispar* Goldschmidt (1911 to 1934) crossed races of different sexual potentialities (the weak European race with the strong Japanese one) and obtained a progeny with intersexed individuals of various degrees. This author introduced into the literature the term "intersex" (1915) and also the notion of the turning point, or point of transition. An intersex is an individual which may be described in the sense of Goldschmidt as a sexual mosaic in time. This means that during its development it changes from one sex to the other. Goldschmidt also set forth the theory of the quantitative relation of the genes. The alteration of normal sexuality was regarded as the result of a genic disequilibrium between F and M which act with different quantitative power in such a way that the more powerful is the one which determines the final result. In *Lymantria*, sex is determined by the quantitative relation of F and M without alteration in the number of chromosomes. This is the case of *diploid intersexuality* in contrast to the *triploid type* of *Drosophila*.

#### Sex Determination by the Y Chromosomes

At present there are only two cases of this phenomenon known. In the plant *Melandrium* the female individuals are XX and the male XY. Warmke has shown that sex can be determined by the interaction of the chromosomes X and Y. In animals, Humphrey (1945) obtained females YY in the silkworm, the sex depending on the presence or absence of the Y chromosome.

### Sex Determination In Parthenogenetic or Haploid Organisms

Among the Hymenoptera (bees, wasps and ants) there are produced haploid males and diploid females, derived respectively by parthenogenesis and fertilization. Sex is determined by a series of multiple alleles, the genes  $X_1, X_2, X_3$ , and so on. Every heterozygotic individual, therefore, ( $X_1, X_2; X_2, X_3; X_3, X_4$ ; etc.) is a female, every homozygotic individual ( $X_1, X_1; X_2, X_2; X_3, X_3, X_4, X_4$ ; etc.) is a diploid male, and the hemizygotic individuals ( $X_1, X_2, X_3$ , etc.) are haploid males. In *Habrobracon*, Whiting has obtained ten classes of diploid males, ten of haploid males and forty five of females. In Brazil, Dreyfus (1944) investigated a microhy menopteran, *Telenomus farlar* showing it to be a case similar to that of *Habrobracon*.

### Inversion of Sex

Although this is a subject which passes somewhat beyond the domain of cyto genetics, we shall say something about the more interesting examples. In the case of the fish *Lebistes*, Winge has succeeded in experimentally changing one sex into the other by transforming the X chromosomes into autosomes and the autosomes into X and Y chromosomes. Due to the easy crossing over between X and Y and to the fact that the autosomes are heterozygotic for both sex genes, he was able to accumulate in a female (XX) a large number of autosomic masculine genes, in such a way that a male developed, even though it had the female chromosome formula. In maize there exists a case of sex determination by a single pair of genes, produced experimentally from a hermaphrodite in a diecious state. The fishes *Platyposyllus* and *Xiphophorus* have a sex determination so uncertain that it has been thought that external factors of an embryonic or postnatal environmental type (phenotypic or epigenetic) may influence it. The same sort of thing occurs in the amphibia, the sexual differentiation of which may be brought about by both genetic and epigenetic factors, furthermore, it is the natural condition for many races to show sex inversion. Among such animals the two potentialities, female and male, occur in a latent state; they are amphisexual (Witschl). The amphibia are rudimentary hermaphrodites both genotypically and morphologically. Males are heterozygotic for sex even though they lack morphologically the sexually differentiated chromosomes (Saex, Rojas and De Robertis, 1936). But the extreme case of great interest is that of the gephyrean worm, *Bonellia viridis* in which the female measures almost a meter and the male, a few millimeters. The male lives within the oviduct and in the intestine of the female. The larvae which lodge in the oviduct are males, but if they leave it, they are transformed into females. The oviduct secretes a substance which is able to change the female to the male sex. Nowinski (1934) carried out experiments in which he demonstrated that larvae may have their sex changed by the action of an extract from the oviduct.

### Gynandromorphs

These organisms form a mosaic of male and female sexual characters with chromosomes of both sexes in different parts of their bodies. They can be thought of as a genetic mosaic in space (Goldschmidt).

In *Drosophila* a gynandromorph is produced by the elimination of one of the X chromosomes (Fig. 111) during the development of the egg. The earlier this is produced the greater are the differences between the female and male parts in the same individual. Figure 111 shows an individual in which the right half is male and the other half female. In the silkworms and in the bee, gynandromorphs are common.

In summary there are the following main types of sex determination

1 By genic equilibrium between the X chromosomes and the autosomes (many organisms, including man)

2 By the mutual action of the X and Y chromosomes (Melandrium and the amphibian axolotl)

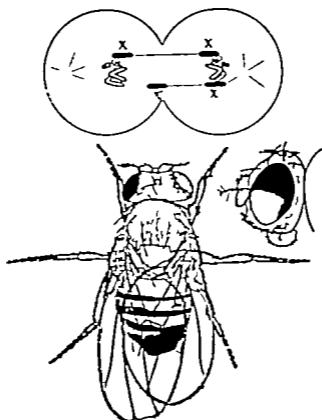


Fig 111 Gynandromorph of *Drosophila*. Above—first division in the segmentation of the egg showing the elimination of an X chromosome. Below—the resulting gynandromorph individual, the left side of which is female (XX) while the right side is male (XO). At the right head of a fly. The X chromosome has been eliminated in one of the last somatic mitoses, showing a red-color spot in the eye (After Morgan, Bridges and Sturtevant taken from Waddington, 1939)

3 By the action of a series of multiple homozygotic and heterozygotic alleles situated at corresponding locations (Hy menoptera)

4 By the differential action of a single gene (some fishes and one case in maize)

5 By the action of the environment (some anuran amphibia and the worm, *Bonellia*)

### Sex Linked Inheritance

X chromosomes have no other genes besides those related to sex determination. These genes are similar to the others found in the autosomes. Particularly in the X

chromosome of *Drosophila* there occur about 500 of these so-called *sex-linked genes*. In crossings these genes follow the distribution of the X chromosome, and the type of inheritance produced is called *sex-linked inheritance*. If a male with red eyes (dominant) is crossed with a female with white eyes (recessive) the offspring of *male sex* have *white eyes* and those of *female sex* have *red eyes*. This is a case of crossed or "crisscross" inheritance (1). If the descendants of this cross are crossed among themselves, one obtains individuals with red eyes and white eyes in the proportion 1 : 1 : 1 : 1 (2)

Parents	$\overline{XX} \overline{XY}$	$\overline{XX}$	$XY$	$\overline{XX} \overline{XY}$	$\overline{XX}$	$\overline{XY}$	$\overline{XX} \overline{XY}$
Offspring	$\overline{XX} \overline{XY}$	$\overline{XX} \overline{XX}$	$XY \overline{XY}$	$\overline{XX} \overline{XY}$	$\overline{XX} \overline{XX}$	$\overline{XY} \overline{XY}$	$\overline{XX} \overline{XY}$
	1	2	3	4	5		

Although the relation 3 : 1 is not observed in the crossing, the second Law of Mendel is still not infringed, as can be seen by following the distribution of the X chromosomes. When a male with white eyes is crossed with a pure (homozygotic) female with red eyes, the sons and daughters will have red eyes (3). If a male with red eyes is crossed with a hybrid (heterozygotic) female with red eyes, there appear three red-eyed to one white-eyed offspring. In this case one of the red-eyed females is pure and the other hybrid, and of the males, one is red-eyed and the other white-eyed (4). In a cross between two progenitors with white eyes (father and mother recessive) all of the descendants (both males and females) have white eyes (5). In man there are known to be some recessive sex linked genes located in the X chromosome having a hereditary distribution exactly like that in *Drosophila*. Among these genes are found those which produce anomalies such as hemophilia, ichthyosis, myopia, the muscular atrophy of Gowers, daltonism and night blindness. These anomalies are transmitted in the same way that the character "white eyes" is transmitted in *Drosophila*. It will be noted that these recessive anomalies are much rarer in women than in men since, for an anomalous son to appear it is sufficient that a woman apparently healthy but who carries the defective gene in a hidden form (heterozygotic) be married to a healthy man (4) such a pair will produce apparently healthy daughters. When an anomalous woman (with two genes in the recessive state) marries (1) a healthy man, the sons will be anomalous and the healthy daughters heterozygotic. If anomalous men marry healthy but heterozygotic women (2) or healthy homozygotic women (3) or anomalous women (5) the results will be those which are indicated in the respective parts of the diagram above. Numerous medical cases which were once obscure have been clarified by this knowledge of the mechanism of sex chromosomes in sex-linked inheritance.

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## Chapter X

### ENZYMES AND CELL RESPIRATION

The diversity of cell functions is extraordinary. In addition to the fundamental activity such as decomposition and synthesis of proteins, fats, carbohydrates, and so on, which characterizes the protoplasm of every cell type, there are in many cases specialized functions such as the production of hormones by endocrine cells or the secretion of sweat by the sudoriparous glands. These general and specialized activities which the cell carries on, and which as a whole are termed *metabolism*, lead one to believe that the cell must have a most complex submicroscopic and chemical organization.

The biochemist Hofmeister calculated the number of molecules which are found in a hepatic cell, arriving at the following figures

225,000,000	million molecules of water
53,000	million molecules of proteins
166,000	million molecules of lipids
2,900,000	million molecules of smaller size

This rough calculation, although instructive, does not convey a true picture of the extreme complexity and organization of a cell, nor does it indicate the chemical changes cells undergo as their functional activity varies.

The cell has been compared to a minute laboratory capable of carrying out synthesis and breakdown of the various substances at normal body temperature. Many of these same processes can take place under laboratory conditions but they usually require high temperatures, high pressures or other extreme conditions. In the cell these chemical reactions are carried out by the intervention of enzymes, which are biological catalysts capable of speeding up or slowing down chemical reactions necessary to vital activity. Enzymes have in this respect certain points of similarity to inorganic catalysts.

Enzymes are conventionally named by adding the suffix *-ase* to the name of the substance on which they act. Thus the enzymes which break down the molecules of proteins are called proteinases, those which act upon phosphoric esters are phosphatases, and so on. In some cases the denomination refers to an entire group of enzymes; for example, all of the enzymes capable of separating hydrogen from various substrates are called dehydrogenases. In

this group succino-dehydrogenase is the enzyme which activates hydrogen from the succinic acid. This terminology also embodies the concept that the enzymatic activity is specific, i.e., each enzyme is capable of acting upon a determined substrate. There are, however, different degrees of specificity. This is 'absolute' when only one definite substrate is attacked, 'stereochemical' where action depends on stereochemical configuration (i.e.,  $\beta$ -glucosidase), or 'relative' when a variety of compounds are split (i.e., lipase).

Enzymes are complex proteins which form colloidal solutions when dissolved. Several enzymes such as urease, pepsin, trypsin, catalase and ribonuclease have been purified and crystallized. The molecular weight of pepsin is 35,500 and of urease, 483,000. Enzymes, like other proteins, behave as zwitterions and have definite isoelectric points. Their activity is influenced by different factors such as the concentration of the substrate and of the enzyme. Temperature increases the rate of reaction up to a certain limit. Enzymic processes also depend on the pH, and for each enzyme there is an optimal hydrogen ion concentration.

Several enzymes, called zymogens, are found in the cell in inactive forms. Zymogens are then activated by substances called kinases. For instance, trypsinogen, produced by pancreatic cells, is activated by enterokinase in the intestinal cavity.

A wide variety of inorganic and organic compounds may influence enzyme activity. Some of them, called *coenzymes*, are diffusible and heat stable substances of small molecular weight which, when combined with an inactive protein component (apoenzyme), form an active complex (holoenzyme). Adenylic acid, glutathione, riboflavin and coenzymes I and II (see Chapter II) belong to this group.

The total quantity of enzymes in a cell varies from one tissue to another. Some estimations indicate that in a single cell there are at least a thousand different enzymes. We must also bear in mind the possibility that, according to the circumstances, a given enzyme may act at one time as a hydrolytic enzyme and at another time show synthesizing activity. This problem will be treated later on.

## METHODS

In early days, biochemists limited themselves largely to analysis of living material in order to learn the various substances which compose it and the proportions in which they occur in the body. Such analysis was based on the mechanical destruction of the entire organ or of smaller or larger pieces of tissues. This

type of procedure was fruitful for its day but could not give us a very satisfactory knowledge of the composition of a single cell nor of the intimate metabolic processes occurring in the cell. One aim of modern biology is to discover how a cell functions, i.e., under which conditions it carries on its vital processes, how it synthesizes its own substances and how it reproduces itself. One approach to these problems lies in the studies of living cells and tissues.

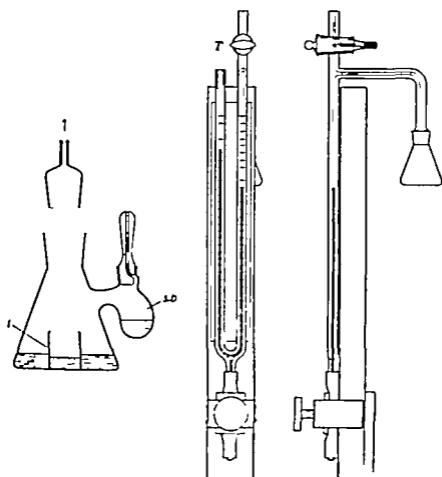


FIG. 11.—Left: Standard reaction flask for Warburg's manometer: *t*, small receptacle (for KOH); *b*, lateral bulb. Right: Barcroft Warburg manometer, seen in front and lateral views. (After Dixon.)

### Warburg's Manometric Method

A great contribution in this direction was made by the German physiologist, Otto Warburg, who, in 1923, described his manometric method.

Nevertheless, with the progress of chemical methods, it has become possible to measure minute quantities of material (microchemistry) and to demonstrate processes in only a few milligrams of tissue. On the other hand, certain histochemical methods mentioned in various chapters of this book permit us to localize and even determine the quantity of substances in the interior of a single cell. We should see these and similar methods in the course of this chapter.

metric method for studying the metabolism of living cells under physiological conditions

As shown in Figure 112, the Warburg apparatus consists essentially of a small reaction flask connected to a manometer. The tissue is placed in the flask and is immersed in a physiological salt solution. The manometer measures the production or consumption of gases by the tissue slices. The whole apparatus is immersed in a constant temperature bath and shaken continuously in order to maintain uniform conditions inside the flasks.

One can illustrate the working of the Warburg apparatus by a concrete example. Very fine slices of recently removed liver are cut and placed within the flask in a physiological solution. Potassium hydroxide is put inside the small chamber situated in the middle of the flask. Through the manometer and the flask a stream of pure oxygen, or a mixture of oxygen and nitrogen, is passed, the stoppers are closed and the

TABLE XII  
OXYGEN CONSUMPTION QUOTIENTS OF VARIOUS TISSUES  
(After Krebs, 1938, modified.)

Organ	Species	$Q_{O_2}$
Erythrocytes	Rabbit	-0.61
Skin	Man	-1.0 to -3.8
Pancreas	Dog	-3.4
Thymus	Rat	-5.4
Submaxillary gland	Man	-6.15
Thrombocytes	Rat	-6.5
Liver	Rat	-8.8 to -13.2
Leucocytes	Rat	-9.1
Thyroid	Dog	-9.1
Adrenal Gland	Rat	-10.0
Kidney (Medulla)	Man	-10.0
Bone Marrow	Rat	-10.5
Cerebrum (Cortex)	Rat	-10.7
Spleen	Rat	-11.8
Hypophysis	Rat	-12.0
Gastric Mucosa	Man	-19.6
Retina	Rat	-30.7

$Q_{O_2}$  Quantity of oxygen consumed per 1 mg. of dry tissue per hour

whole system immersed in the bath. After a few minutes, during which the internal and external temperatures come into equilibrium, the initial reading of the manometer is made. In the interior of the flask the slices of liver consume oxygen and eliminate carbon dioxide. The latter however is absorbed by the potassium hydroxide. As the quantity of oxygen diminishes, the manometer shows changes in pressure. These changes are read at appropriate intervals during the experiment. The quantity of oxygen in cu. mm. consumed by the slices can be calculated from the constants of the manometer and flask.† From the figure reached as a result of these calculations and the dry weight of the tissue, the so-called quotient ( $Q$ ) can be obtained. This quotient expresses the quantity in cu. mm. of gas consumed (or produced) by the tissue per milligram of dry tissue per hour (Table XII).

Warburg used a modified type of manometer previously described by Barcroft.

† In the formula for the calculation of the constant, the reduction to absolute temperature and atmospheric pressure is taken into account.

Other metabolic processes can be studied in a similar way. If one wishes to study anaerobic glycolysis, one can pass an inert gas (nitrogen or a mixture of nitrogen and carbon dioxide) and add an excess of sodium bicarbonate to the physiological salt solution. The lactic acid formed as the final product of this reaction combines with the bicarbonate to form sodium lactate, liberating carbon dioxide. Hence, the manometers show a positive pressure and from the amount of carbon dioxide formed, the intensity of fermentation can be measured.

Numerous and important discoveries have been made by the use of this method and, as a consequence, our knowledge of the function of the enzymes in living systems has made considerable progress.

### Micromanometric Method

In recent years manometric methods have been developed so as to detect very small changes in gas volume, and hence to study the metabolism of minute quantities of tissue (Linderström Lang and Holter, Boell, Needham and Rogers). This ultramicromanometer is based on the fact that a small container or vessel, provided with a bubble of gas and introduced into a larger one containing a fluid of a certain density tends to float, and its vertical position depends on the pressure exerted on the fluid around it. When the outer pressure increases, the vessel sinks, and when it diminishes, the vessel rises. This is the same principle used in the toy called the Cartesian Diver in which the small vessel is represented by a figure of a diver floating in a small jar the top of which is covered by a rubber membrane. When a finger presses the membrane, the diver submerges, but as soon as the pressure is released, the diver rises again. If the pressure is constant, the figure remains in a position of equilibrium. On the other hand, if the external pressure is constant but the quantity of gas inside the diver changes as a consequence of reactions taking place in the interior, the position of the diver also changes and it is necessary to exert or release the pressure to bring the diver to its former position.

The diver as described by Boell, Needham and Rogers (Fig. 113) has in its lower part a "tail" of glass which causes it to maintain a vertical position. Above this is the bulb or reaction chamber at the bottom of which is placed a tiny fragment of tissue or a cell, submerged in a physiological salt solution. Then comes the space for the gas, which occupies part of the bulb and about half of the neck of the diver; the neck is closed by a drop of oil. The diver is submerged in a vessel with a flotation medium which is not excessively viscous and has a minimum solubility for the gas. (Boell, Needham and Rogers use lithium chloride solution, whereas Linderström Lang and Gluck advise ammonium sulfate solution. Perhaps the most suitable solution is one containing a combination of sodium nitrate and sodium chloride.) The entire flotation vessel is connected with a water manometer in which the pressure is kept in equilibrium by means of a syringe provided with screws for coarse and fine adjustments. The pressure is changed until the end of the neck of the diver is opposite to the equilibrium line marked on the outside of the flotation vessel. The pressure differences can be read either directly on the calibrated syringe or on a specially constructed manometer. The quotient is ordinarily  $< 1$ .

culated on the basis of the nitrogen content of the tissue. With this ultramicromanometer it is possible to investigate either the aerobic or anaerobic metabolism, and gas changes of the order of one millionth of a cubic centimeter can be measured. An excellent and detailed description of the theory and technique of the diver method has been published by Holter and Linderstrom-Lang (1943)

Compared with the Warburg method which registers changes of about 20 cu. mm., the diver method shows changes of 0.008 to 0.022 cu. mm. This has made it possible to measure the anaerobic

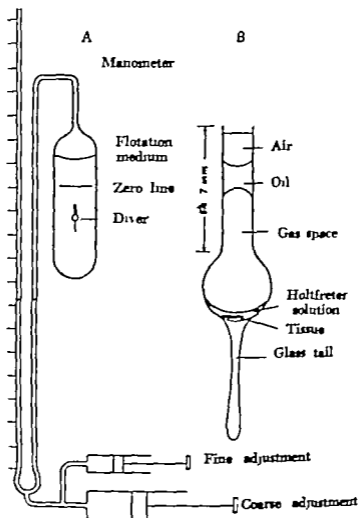


Fig. 113 Schematic view of the ultramicromanometer ("diver" method) A, the manometer B enlarged drawing of the diver (After Boell, Needham and Rogers.)

glycolysis of the dorsal lip of the blastopore in the gastrula of the frog, to demonstrate that it is three times greater than that in the ventral ectoderm, and to determine that the organization center has a greater output of ammonia (Boell, Needham and Rogers). The diver technique has been adapted to the study of respiration of cells in tissue cultures, and it was found that the oxygen uptake of one fibroblast corresponds to  $5 \times 10^{-7}$  ml. (Zamecnik, 1943). On

the other hand, Zeuthen (1946) measured the oxygen consumption of a single frog egg in order to ascertain whether there is a rhythm in the respiration during cleavage, as reported by Brachet and others, who used the less sensitive Warburg method. Holter and Lindahl (1940) with the help of the diver method, investigated the oxygen uptake of animal and vegetative poles of the developing sea urchin egg (*Paracentrotus lividus*). These examples are sufficient to show what an important new field was opened for research, particularly as the diver method is sensitive enough to work with a small group of cells or even a single protozoan.

Based on the principle of the cartesian diver a *diver balance* has recently been developed which permits the weighing of a single amoeba under water (Zeuthen, 1947) and thus permits the following of its metabolic changes through starvation periods of several weeks.

In the majority of cases the manometric methods are used to investigate enzymatic activity indirectly by measuring the amount of gas liberated or taken up in a reaction. There are, however, other methods in which enzyme activity can be measured directly.

### Microanalytical Methods

Many ingenious methods have been devised in recent years whereby quantitative analytical procedures of ordinary chemistry have come to be adapted to measure extremely small quantities of substances. Thus the measurement of 0.1 to 1  $\mu\text{g}$  of nitrogen within an accuracy of 0.005  $\mu\text{g}$  is possible (Brüel et al.) and similar microanalytical methods for potassium and phosphate have been devised. A number of micromethods applicable to enzyme systems are also available.

The use of such micromethods is particularly illuminating when chemical analysis or enzymatic determinations are combined with cytological methods. In this case a correlation between cell function and chemical topography can be obtained.

A detailed description of these methods is beyond the scope of this book (for details see the chapter by Holter and Landerström-Lang, 1940 in the *Handbuch der Enzymologie*). We shall limit ourselves to a very general description only.

In order to obtain the material, the organ or tissue (for example liver or gastric mucosa) is frozen and a cylindrical piece of known diameter is removed by means of a hollow punch. This frozen cylinder of material is cut on a specially oriented microtome (Fig. 115) in sections 10 to 50  $\mu$  thick. Knowing the diameter and the thickness of the sections, the volume of the tissue can be readily calculated. A section is placed in a small reaction tube (of which the total volume is only 0.25 ml.) and then the substrate and other substances are introduced by means of

automatic micropipettes which permit the measurement of quantities of the order of 0.030 cu. mm.

For the titrations, a series of ultramicroburettes is used (Fig. 114) which are composed of four parts (a) a small platform which can be displaced upward and downward and upon which the minute tube for the titration is placed, (b) a microburette with the titration reagent (P) connected with (c) a syringe filled with mercury and provided with a micrometric screw (S) and (d) an electromagnet (A). The titration is carried out in the following way. By removing the screw a small quantity of the reagent is introduced into the tube which contains the product of the enzymatic activity. In order to insure good mixing, a small glass ball filled with iron dust is introduced into the tube. This ball is agitated by the electromagnet.

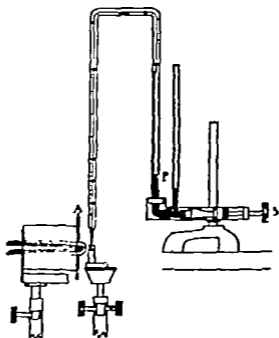


Fig. 114 Diagram of the ultramicroburette. P ultramicroburette. A, magnet, S, micrometric screw. (After Holter and Linderström-Lang.)

These methods have permitted the determination of a series of enzymes in extremely minute quantities of tissue. It was possible, for instance, to demonstrate that in the eggs of various marine animals, peptidase (an enzyme which splits the peptide link) is located in the matrix or fundamental cytoplasm of the cell. Furthermore, Holter, Lanz and Linderström Lang showed that, during the first stages of the cleavage of a developing egg of *Psammochinus miliaris*, peptidase is equally distributed between the animal and the vegetative poles and between the right and left blastomeres.

Another great advantage of these methods is the fact that the biochemical determinations can be made simultaneously with the histological and cytological analysis of the material. For this purpose, alternate sections of the plug of tissue can be fixed or

stained for microscopic study (Fig 115) and the results correlated with enzyme activity of the slice (see, for instance, Landerström Lang and Mogensen)

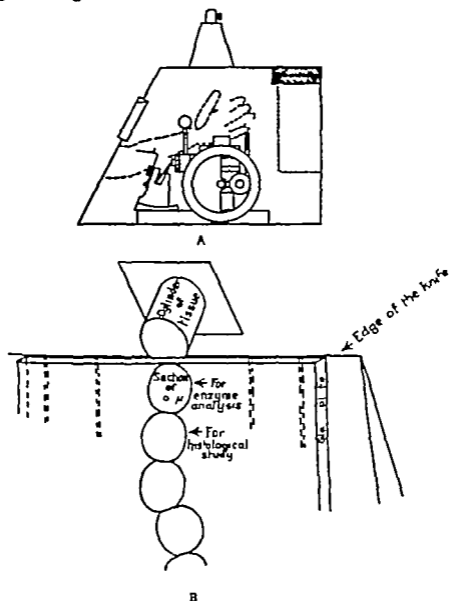


Fig 115 A, Microtome for frozen sectioning. The apparatus is kept in an especially refrigerated container which keeps the tissue and the sections frozen. B Scheme of the sectioning with the freezing microtome. The cylinder of tissue is sectioned and the sections are collected so that each alternate piece is used for enzymatic analysis and the others for histological control. (After Landerström Lang)

## LOCALIZATION OF ENZYMES

### Topography of Enzymes in Tissue

As an example of localization of enzymes in tissue we shall consider in detail the work done on proteinases in the gastrointestinal tract. Landerström Lang and his collaborators have

studied the localization of pepsin in different regions of the stomach and the juxtapyloric portion of the duodenum of the pig. For these estimations, the gastric mucosa was frozen and cylindrical plugs 2 mm. in diameter were cut from different zones. These cylinders were then sectioned on a freezing microtome in planes parallel to the surface of the mucosa. In the series of

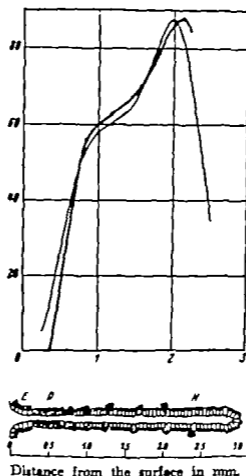


Fig. 116 Pepsin content of the various parts of a fundic gland from the stomach of the pig. The coarse line represents the quantity of pepsin (in units) at various distances from the surface of the mucosa; the fine line represents the number of principal cells at the corresponding locations. In the lower part of the figure, a diagram of a fundic gland of the pig the depth of which (in mm) coincides with the corresponding divisions of the curve. (After Holter and Linderström-Lang, modified.)

sections obtained, the degree of pepsin activity was determined according to the methods described above. Thus the enzymatic activity could be demonstrated to be dependent on the localization of the section of the mucosa (Fig. 116). These studies have a particular histochemical importance because they correlate the activity of the enzyme in different sections with the histological picture of adjoining sections. It was shown that the content of pepsin in the cardiac region and duodenum is very low. The

greatest activity was found in the fundus at a distance of 2 to 2.5 mm. from the surface, which coincides with the sites of greatest abundance of the zymogenic (chief) cells (Fig. 116). The pyloric region also has its maximum activity at the same level in the mucosa, but here the activity is much less than in the fundus.

It has to be stressed that pepsin does not occur as such in the cells but in an inactivated form called pepsinogen. At pH 2.0 pepsinogen is converted into pepsin. However the experiments of Linderström Lang and his collaborators have been described in terms of proteolytic activity as pepsinogen does not hydrolyze proteins.

TABLE XIII  
CONTENT IN PEPSIN AND DIPEPTIDASE OF CELLS IN  
DIFFERENT PARTS OF DIGESTIVE TRACT  
OF THE PIG  
Activity Expressed in Enzyme Units Multiplied by 1000  
(After Linderström Lang and Holter 1940 modified)

	Cardia		Fundus		Pylorus		Duodenum	
	D	P	D	P	D	P	D	P
Epithelial cell	(0)	+	(0)	0.2	(0.03)	0.07-0.11		
Neck chief cell	(0)		(0)	0.2-1.2	0.23	0.09-0.16		
Chief cell		-	(0.06)	1.7-2.1	0.2	0.14-0.23		
Goblet cell							(0)	±0
Cylindrical cell							(0.64) 0.81	
Epithelial cell			(0)	(0)				
Brunner cell							(0.2)-0.32	0.04

D: Dipeptidase P: Pepsin. Figures in parentheses represent uncertain results

In Figure 116 the pepsin content of the fundic region of the stomach in the pig is plotted as a function of the relative number of cells. The abscissa shows the distance in millimeters from the surface while the ordinate shows the quantity of units of pepsin for each section 50  $\mu$  in thickness. The finer line corresponds to the proportion of chief cells and the coarser line to the pepsin content. The two curves coincide. This is evidence that the chief cells are those which contain and produce pepsinogen.

On the basis of these studies, it was possible to calculate the pepsin content of single cells of various regions of the glands. Table XIII shows the amounts of dipeptidase and pepsin in different cells and regions of the digestive tract. From this table it follows that there is very little pepsin produced in the epithelial cells and the chief cells of the cardia whereas the greatest amount

of this enzyme is located in the neck chief cells, epithelial cells and chief cells of the fundus and pylorus. On the other hand, Brunner's cells in the duodenum are rich in dipeptidase but contain little pepsin. In contrast, the epithelial, neck chief cells and chief cells of the fundus are very rich in dipeptidase. These same groups of cells in the pylorus contain (with the exception of the epithelial cells) a fair amount of both enzymes. The table demonstrates further on that the chief cells and the neck chief cells of the fundus are primarily responsible for the production of pepsin, whereas the same cells similarly situated in the duodenum are the chief producers of dipeptidase.

TABLE XIV  
LOCALIZATION AND APPROXIMATE CONCENTRATIONS OF SOME ENZYMES IN  
LIVER CELL NUCLEI  
(From Dounce, 1947 modified.)

Enzyme	Present or Absent in Liver Cell Nuclei	Approximate Concentration Expressed in Per Cent of Concentration in Cytoplasm
Alkaline phosphatase	Present	10%
d-amino acid oxidase	Present	100
Arginase	Present	40-50
Esterase	Present	50
Acid phosphatase	Present	25-30 or higher
Cytochrome oxidase	Present	
Cytochrome c	Low	
Succino-dehydrogenase	Low or absent	
Coenzyme I	Low	
Choline oxidase	Absent	

In regard to the content of the proteases in other regions of the digestive tract, it is known, for example, that in the rat the dipeptidase is found in greatest concentrations at the level of the villi and in the region where the cells of Paneth occur. According to Van Weel and Engel, this enzyme is secreted by the cells of Paneth and adsorbed at the surface of the villi and, hence, greater quantities of dipeptidase are found.

The problem of localization of the proteolytic enzymes in the pancreas in relation to the significance of secretion granules will be dealt with in Chapter XI.

### Intracellular Topography of Enzymes

The topography of enzymes within cells is little known. Early investigators considered the nucleus as the metabolic center of the cell, particularly in relation to cell respiration and the

localization of enzymes. This hypothesis has not been corroborated by modern work, on the contrary, the nuclear oxygen consumption and enzyme content proved to be in general much lower than those of the cytoplasm. In germinal vesicles of frog eggs removed mechanically oxygen consumption of only 1 to 2 per cent of that of the total egg has been found (Brachet). Analyses of enzyme content have been carried out on nuclei of liver cells, isolated and concentrated by techniques which involve fragmentation of the cell and differential centrifugation (Fig. 54) (See Chapter VII). Table XIV shows some of the results obtained. It can be readily seen that the only enzyme present in liver nuclei in a higher concentration than in cytoplasm is alkaline phosphatase. This fact is confirmed by Gomori's histochemical technique. In isolated nuclei, metabolites are generally absent

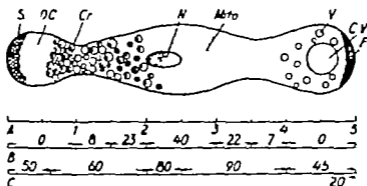


Fig. 117 Schematic view of an amoeba after being subjected to the action of centrifugation. DC, dense matrix; Cr, crystals; N, nucleus; Mito, chondriosomes; V, vacuoles; CV, contractile vacuole; F, flagellum. Scale A represents the divisions of the body of the amoeba into five sectors. Scale B represents the percentage of chondriosomes in the different parts of the amoeba. Scale C shows the content of matrix in different sectors. (After Holter and Doyle.)

and coenzymes are present only in low concentrations. These results may be due to an original low concentration or to losses during the preparation of the material. By this technique also the following vitamins have been found to be present in the nucleus: riboflavin, inositol, nicotinic acid, pantothenic acid, thiamine, pyridoxine, folic acid and biotin (Dounce).

The intracellular localization of *proteolytic enzymes* has been studied in the eggs of the sea urchin, *Dendraster excentricus*. In these eggs the nucleus has an eccentric position and, hence, if

Phosphatases are enzymes involved in the breakdown of phosphoric esters with liberation of phosphate ions. The histochemical technique involves the incubation of tissue sections with a substrate containing glycerophosphate. Phosphoric acid liberated in the reaction is immediately precipitated as calcium phosphate by calcium ions also present in the substrate. The precipitated phosphate is then made evident by treatment with cobaltous salts and ammonium sulfate (Gomori).

the eggs are divided into two portions, one half can be obtained containing the nucleus and the other only cytoplasm. It was found that the parts with the nucleus show greater peptidase activity. From these experiments it seems probable that this enzyme is bound up, at least in the greater part, with the nucleus. The same problem was studied in the amoebae. These Protozoa can survive without a nucleus for three days. Holter and Kopac removed the nucleus surgically from amoebae and compared the activity of denucleated animals with that of normal ones. In this case, however, they did not find differences in activity between enucleated and nucleated amoebae.

TABLE XV

DISTRIBUTION OF ENZYMES IN FRACTIONS OF LIVER HOMOGENATES OF THE RAT  
(From Schneider 1946, modified.)

Tissue Fraction	Succino- oxidase	Cytochrome Oxidase	Adenosine Triphosphatase
Original Homogenate	383	1012	865
Nuclear Fraction	25.4	51.6	231
Mitochondrial Fraction	280	748	416
Unfractionated Residue	45.5	147	257

Th activities are expressed as mm. of O<sub>2</sub> taken up per 10 minutes by the equivalent of 100 mg. of fresh tissue.

†Th activities are expressed as micrograms of phosphorus liberated in 15 minutes by the equivalent of 100 mg. of fresh tissue.

We shall take as a further example the localization of *amylase* (the enzyme which hydrolyzes starch) in *Amoeba proteus* (Holter and Doyle, 1938). When these Protozoa are centrifuged, their components are separated and stratify in the form shown in Figure 117. It can be noticed that the chondriosomes (*Mito*) and nucleus (*N*) settle at about the middle of the body. At the centripetal end are found drops of lipids (*F*), the contractile vacuole (*CV*) and minute vacuoles (*V*). At the centrifugal end are accumulated some inclusions (*S*, *Cr*) and the dense cytoplasm or matrix (*DC*). Under these conditions the amoebae were sectioned in five planes represented on the line *A* and the volume of the fragments calculated by the space which they occupied on being aspirated with a calibrated pipette. On the line *B* the percentage of the chondriosomes in each one of the regions is indicated, and on the line *C* the percentage of the cytoplasmic matrix. Though these experiments have not shown very definite differences in amylase activity of the various regions, it is nevertheless evident that the parts which contain a greater percentage of mitochondria

show also a more intense enzymatic activity. The authors therefore draw the conclusion that, with all probability, amylase is localized in the chondriomes of the amebae. Using a similar technique, Holter and Kopac (1937) demonstrated that the peptidase in ameba is localized in the matrix.

Recent investigations carried out mainly on liver cells, by using the technique of fragmentation and differential centrifugation, suggest the conclusion that some of the important enzymes involved in cell respiration are associated with relatively large granules (0.5 to 2  $\mu$  in diameter) present in the cytoplasm and generally considered to be mitochondria.

Table XV shows that succino-oxidase and cytochrome oxidase are very definitely associated with the mitochondrial fraction. For example, in the case of the cytochrome oxidase, calculating in percentages the results shown in Table XV it is found that the mitochondria contain 73.9 per cent of the original enzyme activity while the nuclei contain only 5.4 per cent and the unfractionated residue 14.5 per cent.

Similar results were obtained after a more complete fractionation of the cytoplasmic homogenate.

TABLE XVI  
DISTRIBUTION OF CYTOCHROME OXIDASE AND SUCCINO-OXIDASE IN CYTOPLASMIC  
FRACTIONS OF THE RAT LIVER  
(From Hogeboom, Claude and Hotchkiss, 1946, modified.)

Liver fraction	Cytochrome Oxidase Per Cent Recovery	Succino-oxidase Percentage Recovery
Liver extract	100	100
Large granules	70	1
Microsomes	<4	-
Supernatant	0	0

A typical example is seen in Table XVI which shows the strongest succino-oxidase and cytochrome oxidase activity to be present in the large granules fraction (mitochondria). It is also seen that microsomes (see Chapter V) contain a small proportion of activity which probably can be accounted for by contamination with large granules or large granule fragments, and that the supernatant left after sedimentation of mitochondria and microsomes shows no activity whatever. These results tend also to indicate that the respiratory enzymes are bound to mitochondria.

Recently cytoplasmic granules were separated by centrifugation in five fractions of different granule size. It was found that

alkaline phosphatase and adenosine pyrophosphatase is more concentrated in the larger granules. In contrast, the ribonucleic acid is much more concentrated in the smaller granules (Chantrenne, 1947)

## Enzymes and Cell Structure

The data we have discussed on the intracellular topography of enzymes lead us to consider the relation between cell structure and enzyme activity

It is necessary to recall that, referring to their site of action, there are different classes of enzymes. Some, as for example, pepsin, trypsin and ptyalin, are produced by the cell in an inactive form and are secreted into the cavities of the body where they become active. Other enzymes are secreted by the cell into the intracellular tissue spaces (i.e., hyaluronidase) and finally many enzymes are localized entirely within the cell and serve to maintain all the vital processes. In this section we shall discuss exclusively the last group.

Among these intracellular enzymes there is a series which can survive destruction of the cell. Such is the case with enzymes of yeast for the alcoholic fermentation. A cell free yeast extract is capable of fermenting sugar although it is somewhat less active than the living cell itself. Other enzymes such as ascorbic-acid oxidase and ribonuclease, also act when extracted from the cell. There are, however, other intracellular enzymes, the activity of which ceases as soon as the cellular organization is destroyed. Thus, for example, the cytochromes *a* and *b* (about which we shall speak later) lose their activity when the cell is destroyed, whereas cytochrome *c* is easily extractable.

These facts lead to the distinction of two types of enzymes: those which do not need the structural unity of a cell and, therefore, can act in homogenates, and those which are intimately bound up with the morphological organization of the cell.

The degree of union of the enzyme to the protoplasm seems also to have an important influence on the way the enzyme acts. Oparin claims that some hydrolytic enzymes have the capacity of either synthesizing or hydrolyzing, according to the degree of fixation in the protoplasm of the cell.

Willstätter and Rohdewald distinguish three types of enzymes: (1) the *lyoenzymes* or enzymes dissolved directly in the protoplasm, the extraction of which is relatively easy; (2) the *desmoenzymes* which, in contrast to the former, are bound to the protoplasm and therefore cannot be extracted by the available

methods and (3) the *endoenzymes* which are apparently adsorbed or bound to the plasma membrane, whose extraction is possible only when the membrane is destroyed by chemical or mechanical means

An attempt to analyze the relation between cell structure and respiratory enzymes was carried out by correlating oxygen consumption and succino-dehydrogenase activity with cytological structure both in sections and in liver homogenates. This study was done on normal tissue or after freezing in liquid air or freezing and drying in vacuum. (De Robertis and Nowinski, 1942)

TABLE XVII

COMPARISON BETWEEN CYTOLOGICAL CHANGES, OXYGEN CONSUMPTION AND SUCCINO-DEHYDROGENASE ACTIVITY IN LIVER TISSUE  
(Nowinski and De Robertis, 1943.)

Cellular Structure	Normal		Frozen		Frozen Dried	
	Tissue	Homogenate	Tissue	Homogenate	Tissue	Homogenate
Cell boundaries	Normal		Apparently normal		Apparently normal	
Aspect of the cytoplasm	Normal		Vacuolated		Vacuolated	
Nuclei	Normal	Present	Shrunk and pyknotic	Present	Shrunk and pyknotic	Present
Chondriome	Normal	Present	Lacking	Lacking	Lacking	Lacking
Oxygen consumption ( $Q_{O_2}$ )	-6.47	-2.01	-2.04	-1.72	-1.41	-0.99
$Q_{O_2}$ with sodium succinate	-8.21	-6.57	-6.38	-2.89	-1.50	-1.10

The main results are summarized in Table XVII in which are given the most important cytological changes along with the oxygen uptake ( $Q_{O_2}$ ) without and with sodium succinate as substrate.

It may be noted that freezing considerably diminishes the oxygen uptake—about 68 per cent of that of the tissue. This result seems to be due, in part, to the diffusion of the substrates out of the cell, as a vacuolization can be observed histologically and the addition of the succinate increases the consumption of oxygen considerably (from -2.04 to -6.57).

If liver homogenate is observed under the microscope, some parts of the cells, such as the nuclei and the chondriosomes, are

often intact (see Table XVII) If, however the homogenate is frozen, the chondriosomes disappear and at the same time the activity of the succino-dehydrogenase decreases considerably (from  $Q_{O_2}$  -6.57 to -2.88)

These results were interpreted as indicating that the activity of the succino-dehydrogenase is linked to the presence of the chondriosomes as an intracellular unity. It is interesting that recently it has been proven that this enzyme is present in high concentration in the mitochondrial fraction (see above). It seems very possible that the disruption of the molecular organization of these organoids by freezing affects the activity of the enzyme.

The study of the intracellular localization of enzymes and their relation to cell structure and function has only started. As Green (1937) points out, the major discoveries of the mechanisms which cells utilize for their reactions have practically all been made by the analysis of the behavior of cell extracts and of enzyme systems but "the materials of the cell offer unlimited possibilities of combination and interaction, few of which are realized by the cell under normal conditions. He therefore emphasizes that such phenomena have no biological importance unless a similar process is observed *in vivo*. This, of course, is perfectly justified and true as far as enzymatic systems, like the Warburg Keilin system of cellular oxidations or the process of phosphorylation, are concerned. Also Commoner (1942) who extensively discussed the problem of cell structure and oxidations, concluded that the most important factor determining the course of chemical events in the cell is the very structure which is destroyed in the process of extraction.

The study of tissues, homogenates, or even of mistreated sections, in which the cells can be easily damaged, is often misleading. Such preparations may show many reactions similar to those present in the normal tissue, but especially those pertaining to synthetic processes have disappeared (Krebs). In a ground tissue, although some cytological structures such as the nucleus and the mitochondria may persist, with the disintegration of the plasma membrane, the displacement and dilution of the structural proteins, oxidation reduction systems, metabolites and electrolytes, the whole molecular organization of the cell is destroyed.

Furthermore, cellular damage brings forth enzymatic processes which in the intact cell are kept in check for example, the destructive action of phosphatase on phosphorylated compounds, the destructive action of the pyridine nucleotide-splitting enzyme which destroys the enzyme components, diphospho- and triphosphopyridine nucleotides. The destruction of the cellular

architecture, and of the semipermeable membranes produces, also changes in the orientation of reactions and brings forth stimulating or inhibitory effects of the electrolytes dissolved in the tissue suspension (Guzman Barron)

It is evident that the exact knowledge of how metabolic processes occur inside the cell and are regulated to follow definite orientations will only be attained by the integrated studies of the molecular organization of the protoplasm, which constitutes the basic machinery of the cell, plus the investigation of how enzymes and metabolites are localized and coupled to that machinery to bring about the orderly processes characteristic of life.

### CELL RESPIRATION

The oxidation of metabolites furnishes the cell with the energy required for its numerous functions. This oxidative process which generally involves the utilization of oxygen and the production of carbon dioxide and water is referred to as *respiration*.

For a long time it was thought that oxidation and its opposite process, reduction, were based only on the direct chemical combination with oxygen or the loss of this element. It is now known, however, that under anaerobic conditions within the cell, in spite of the fact that there is no molecular oxygen in the system, a series of oxidations and reductions may take place.

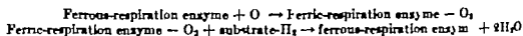
The modern concept of oxidation reduction is based on the atom model and defines oxidation as the withdrawal of electrons from a molecule (or atom) and reduction as the addition of electrons (Michaelis)

Therefore, besides the direct combination with oxygen, at the present time the following chemical changes are considered as oxidations (a) The loss of hydrogen for example, ascorbic acid is oxidized to dehydro-ascorbic acid by the loss of two atoms of hydrogen (b) the addition of water with the simultaneous loss of hydrogen, as in the case of the formation of acetic acid from ethyl alcohol (c) the loss of electrons without simultaneous addition of  $O_2$  or loss of  $H_2$ . For example bivalent iron, Fe (ferrous) is oxidized into trivalent iron, Fe (ferric). However in all such chemical processes the fundamental fact of oxidation is the loss of electrons by the molecule or atom oxidized.

In a similar way the reverse processes of reduction take place by addition of electrons.

Much of our knowledge of the mechanism of cellular oxidations stems from investigations by Warburg of the catalytic activity of organically bound iron. He found that cell respiration is inhibited irreversibly by cyanide and reversibly by carbon

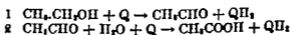
monoxide in the dark. Since it was known that iron heme compounds possess this property and that they readily combine with cyanide (as in the case of hemoglobin) Warburg deduced that there must exist in the cell an enzyme containing iron, capable of combining with oxygen in normal circumstances. Spectroscopic analysis revealed this compound, which he called the respiratory enzyme ( *Atmungsferment* ) and which he found to be related to hemin. Since this respiratory enzyme behaves similarly to hemoglobin, it was concluded that the active part of the molecule was iron and that this enzyme was carrying out its function by activating the oxygen and transferring it to another compound. Schematically, the reaction may be expressed as follows



This scheme not only represents Warburg's idea of the action of the respiratory enzyme, but it also corresponds to the concept of the mechanism of enzymatic reactions which is in use today

When Warburg was working out his theory of cellular oxidations, Wieland published an opposing view based on a principal role played by hydrogen rather than by oxygen. He demonstrated that oxidations could occur in the complete absence of oxygen (namely by the loss of hydrogen) when there exists in the system a substrate capable of giving up hydrogen (in the presence of a suitable catalyst called a dehydrogenase) to a suitable hydrogen acceptor

Wieland studied under anaerobic conditions the oxidation of alcohol by bacteria, first to acetaldehyde and then to acetic acid. Using a quinone as the hydrogen acceptor he obtained an oxidation of alcohol as complete as in aerobiosis. These results were particularly striking since the acetic acid ( $\text{C}_2\text{H}_4\text{O}_2$ ) has not only two hydrogen atoms less than ethyl alcohol ( $\text{C}_2\text{H}_6\text{O}$ ) but also one additional oxygen atom. The source of this oxygen in a reaction taking place presumably in the absence of oxygen was found by Wieland to be due to the separation of hydrogen and the addition of a water molecule. The reaction postulated involves a hydrogen acceptor (Q) and takes place in two steps



An example of a substance commonly employed as a hydrogen acceptor is methylene blue. This dye, which in its oxidized form is blue, by combining with hydrogen is reduced to its colorless form (leucoform)

In order to investigate enzymes capable of activating and removing hydrogen atoms (dehydrogenases) it is convenient to incubate homogenates at body temperature and to do this under strict absence of oxygen with methylene blue. Decolorization of the dye thus indicates the course of the reaction, and the time in which decolorization takes place is a measure of the dehydrogenase activity (Thunberg).

From such experiments new concepts were introduced which are of great importance in the interpretation of oxidations and reductions. These include the concepts of acceptors and donors of hydrogen or of oxygen. Oxygen itself is an example of a hydrogen acceptor and the ethyl alcohol in Wieland's experiments, mentioned above, exemplifies the hydrogen donor.

The concept of cellular oxidations was very much broadened when, in 1925, Keilin rediscovered a pigment which was later demonstrated in almost all types of cells, with the exception of some anaerobic bacteria. This pigment, which he called *cytochrome*, is an iron compound bound to a protein molecule (ferroporphyrin) in a form similar to hemoglobin. This substance had first been described by Mac Munn in 1886 in various animal tissues, but due to an unjust and violent criticism of some of the most distinguished chemists of that time, it promptly fell into oblivion. Keilin rediscovered it in the thoracic muscle of the bee and observed it under the microscope to which a spectroscopic adapter had been added. Under these conditions, he observed that the pigment is present in a reduced or in an oxidized form which shows differences in absorption bands. Since the reduced cytochrome showed three distinct absorption bands, Keilin thought that there are three different cytochromes, which he designated as *a*, *b* and *c*. Later studies have demonstrated that the three cytochromes possess the following absorption bands:

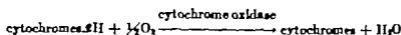
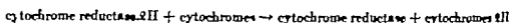
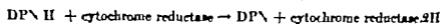
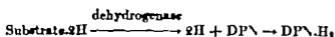
Cytochrome <i>a</i>	6050 Å, 4520 Å
Cytochrome <i>b</i>	5640 Å, 5300 Å, 4320 Å
Cytochrome <i>c</i>	5507 Å, 5—3 Å, 4150 Å

The bands are those of the reduced cytochrome; the bands of oxidized cytochrome are not sharp but oxidized cytochrome *c* has bands at 5300 Å and 4000 Å. Owing to these spectroscopic properties the cytochromes can be observed with the microscope in the living cell, an ideal circumstance for studying their physiological oxidation and reduction.

Each of the cytochromes has different properties. Cytochrome *c* combines with carbon monoxide and with the cyanides ( $\text{KCN}$  or  $\text{HCN}$ ); the latter also inhibit the ferro compounds of cytochrome

end of which is the substrate as a hydrogen donor and molecular oxygen at the other end as a hydrogen acceptor. Hydrogen moves through the series of reactions toward oxygen until they combine to form water.

The following simplified diagram of the hydrogen path may help to follow the respiration cycle:

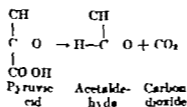


It is interesting to point out in this cycle the intervention of two vitamins belonging to the B complex. Nicotinic acid amide is found in both DPN and TPN and riboflavin (vitamin B<sub>2</sub>) in the cytochrome reductases. Below we will mention other instances in which vitamins are directly linked to enzymes in fundamental metabolic processes.

The described system of oxidations, called the Warburg Keilin system, has been most thoroughly studied and seems to be the most important one. However some other system may exist. We know for instance, that respiration cannot be completely inhibited by cyanide and the problem of this "residual respiration" is not yet properly understood. The fact that neither the flavoprotein enzymes nor cytochrome *b* are inhibited by cyanide may indicate that these could be involved in such "cyanide insensitive respiration."

In the oxidation cycle studied, the end product is water. Let us now consider how carbon dioxide, the other final product of cell respiration, is formed.

Carbon dioxide is mainly derived from the decarboxylation of organic acids resulting from the metabolism of carbohydrates or lipids and also from amino acids. For example, pyruvic acid is transformed in acetaldehyde and carbon dioxide:



This reaction is catalyzed by the enzyme carboxylase, which is activated by a cocarboxylase containing thiamine (vitamin B<sub>1</sub>). Several bacterial and animal decarboxylases act on amino acids.

and are activated by the coenzyme pyridoxal  $\text{PO}_4$ , which is a derivative of pyridoxine (Vitamin  $\text{B}_6$ )

The principal function of oxidative processes is that of supplying the organism with the necessary energy to maintain its vital activities. This chemical energy is then converted into thermal, mechanical, electrical, or other type of energy. When oxygen combines with hydrogen to form water sixty-eight kilocalories per mol are liberated.



If the process of oxidation should consist only of this reaction, the whole amount of energy would be released at once. This is not the case, however, and during the oxidation cycle quanta of energy are released in steps. This way of liberating energy is more convenient to the cell because energy can be fully utilized in the different activities of the cell only when supplied in small amounts. For example, in the synthesis of the energy rich phosphate bond found in adenosine triphosphate and other compounds, eleven kilocalories per mol (of the total of sixty-eight) are necessary. This stored energy can then be released suddenly under the action of enzymes such as adenosine triphosphatase. This reaction seems to be of particular importance in muscle contraction because myosin apparently has adenosine triphosphatase activity and thus can directly split the energy rich phosphate bond. As will be mentioned in Chapter VII this is one of the best examples in which energetic processes are directly linked to the molecular organization of the cell.

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## Chapter XI

### VISIBLE MANIFESTATIONS OF CELLULAR ACTIVITY

The chemical changes and transfers of energy which characterize cell metabolism, although demonstrable by biochemical methods (Chapter V), are generally invisible with the light microscope. They occur on the level of the submicroscopic structure (see Chapter IV) and under the catalytic influence of the intracellular enzyme systems (see Chapter X).

Cell metabolism may nevertheless be made evident by manifestations of energy change or by structural modifications. This may be exemplified by an intestinal cell which absorbs fats as fatty acids and glycerol and later synthesizes them into neutral fats which accumulate in the form of minute refractile droplets. The first two steps, absorption and synthesis are invisible, the last—accumulation—has a microscopic expression. As another example, the hepatic cell receives glucose molecules from the blood and polymerizes them into glycogen which accumulates on microsomes. The aggregates of microsomes are visible with the microscope. Gland cells likewise absorb a series of substances in molecular form which are then transformed and accumulated in granules of secretion which are visible.

In other cases, the liberation of chemical energy is translated into *motor manifestations* such as amoeboid or ciliary movement, or into the production of heat, electricity, light, or other forms of energy.

Finally, cell metabolism is made evident also by *growth division, differentiation* and *repair* of the cells.

In this chapter we shall consider some of these microscopic manifestations of cellular activity. Others are presented in previous chapters and in Chapter XII.

#### Cell Movement

Cell movement is a manifestation of the mechanical energy of the cell and one of the most objective signs of its activity. When we observe the displacement of a cell in a tissue culture, we have the impression that it is actually living; nevertheless vital phenomena may occur without any apparent movement of the protoplasm. In certain cases, cell movement is within the protoplasm and produces no exterior deformation of the cell. Thus

type of motion wherever found is here called *protoplasmic streaming* or *cyclosis*. In other cases, the movement is manifested by the emission of pseudopods and leads to a true displacement of the cell (ameboidism). Furthermore, movements may occur in

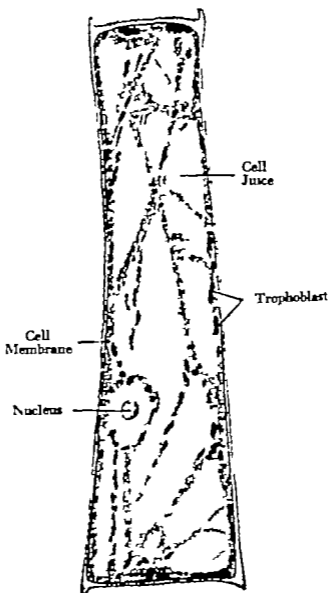


Fig. 118. Cell from an epidermal hair of the pumpkin in the living state. (After Maximow)

specially differentiated appendices—*ciliary motion*—or in specific cytoplasmic fibrillae—*muscular motion*.

### Cyclosis

*Cytoplasmic streaming* or *cyclosis* is easily observed in numerous plant cells in which the cytoplasm is generally reduced to a layer next to the cellulose wall and to fine trabeculae which cross the great central vacuole (Fig 118). In the peripheral cyto-

plasm as well as in the trabeculae, continuous currents can be seen which displace the chloroplasts and also the cytoplasmic granules. In the ciliated Protozoa—such as *Paramecium*—similar but slower movements are seen, which displace the digestive vacuoles from the site of ingestion to that of excretion. In many cells of higher animals and particularly in tissue culture such intracellular movements are seen. In cases where it is difficult to see them, the observation can be facilitated by using a dark field condensor or cinematography. In this type of cell movement is included also the karyokinetic division with its complex displacement of the cell center and of the chromosomes, the changes of position of the chondriome, of the Golgi apparatus and of the nucleus.

The classical experimental work on cyclosis has utilized plant cells. Little work has been done with animal cells. In some plant cells the protoplasmic current is initiated under the influence of a chemical stimulus (*chemodynesis*) or by light (*photodynesis*). It has been observed that the extracts of cells in a state of cyclosis are capable of initiating the movements in cells in repose. According to Fitting this action probably is due to the presence of amino acids of the type of histidine.

Cyclosis is modified by temperature and shows a maximum activity at some optimal temperature. All of the normal or pathological processes which increase cell metabolism also increase the protoplasmic movement. Mechanical injuries or electrical shock may stop cyclosis. It is also modified by the action of ions, alcohol, or by changes in pH. This movement can continue in the absence of oxygen. Some auxins (plant hormones) increase the velocity of cyclosis when they act in small concentrations. In general, all of the factors which decrease cell viscosity increase the velocity of the protoplasmic current, and vice versa (See Chapter III).

It is evident that viscosity is one of the most important factors which intervene in the intimate mechanism of cyclosis, since the protoplasmic colloid may pass by reversible sol  $\rightleftharpoons$  gel changes from a liquid or almost liquid state to one which is almost solid. Nevertheless, the propulsive force of the movement is not well known.

### Ameboid Movement

*Ameboid movement* is a very common mechanical manifestation in animal cells. While in cyclosis the protoplasmic components are simply displaced without any deformation of the cell in this case actual deformation occurs. The cell actively changes

shape, sending forth cytoplasmic prolongations called pseudopodia into which the protoplasm flows. This phenomenon leads to a special form of locomotion called *ameboid* because it finds a very definite expression in the amebae. This type of motion is observed, nevertheless, in numerous types of cells. One need only place a drop of blood between a slide and a coverglass, avoiding drying, and maintaining a proper temperature, to see that the leucocytes, at first spheroidal, change their form, emit pseudopodia and move about. In tissue cultures all of the explanted elements, whether they are mesenchymal, endothelial, epithelial, and so on, may free themselves from the rest of the tissue and move out actively forming the zone of growth (Fig. 11)

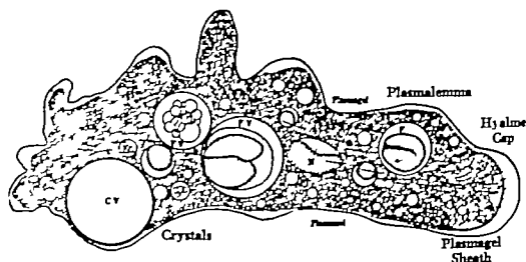


Fig. 119 Structure of an ameba, after a diagram from Mast. CV contractile vacuole; FV food vacuoles.

In many of these cases, the cells must undergo a previous modification, a process of "dedifferentiation," in order to acquire this active ameboid form. This is what happens in the epithelia when the tonofibrils uniting the cells disappear by a process of localized superficial liquefaction. These transformations also occur *in vivo*. For example, in epithelial repair the cells free themselves and slide along actively toward the depth of the wound. In an inflammatory process, leucocytes wander out of the blood vessels (diapedesis) by active ameboid motion and progress toward the focus of infection.

The ameba is the ideal material for the observation of this type of motion. As shown by the classical studies of Mast, the protoplasm of the ameba has a clear ectoplasm, without visible structure, which expands considerably toward the end of the pseudopod (hyaline cap) and a granular endoplasm which constitutes the greater part of its mass. In the endoplasm this author

distinguishes two parts a peripheral part (plasmagel) which is immobile, and a central part (plasmasol) which flows along as the ameba progresses. The plasmagel is lacking at the advancing pole of the ameba or is reduced to a very delicate layer so that the whole may be compared to a sac open anteriorly (Fig. 119). Under these conditions the internal endoplasm (plasmasol) flows into the pseudopod and is followed by the rest of the protoplasm (Fig. 120).



Fig. 120 1-4 Advancing end of a slime mold. Photomicrographs were taken at 10 second intervals without moving the camera. The base line remained constant so that the advance and retraction could be measured. 1 Hyaline cap at the tip of an advancing pseudopod showing the even bulging contour of the granular gel layer. 2 Same tip, 40 seconds later. The thin gel layer has disintegrated and granules are filling the cap. 3 Same tip, 20 seconds later. Gelation of the periphery of the granular protoplasm and the formation of a new hyaline cap. 4 Same tip 30 seconds later. Retraction of the pseudopod and irregular hyaline cap. (Courtesy of W. H. Lewis and the Iowa State College Press. In "Structure of Protoplasm," 1914.)

In leucocytes there is an anterior pseudopodial zone which is highly mobile and consists of plasmasol while the posterior part (in relation to the direction of movement) is rigid and in the gel state. During locomotion the lateral part of the anterior plasmasol gels and becomes immobilized (De Bryun).

The speed of progression varies among different amebae between 0.5 and 4.6  $\mu$  per second. In the neutrophil leucocytes it is approximately 0.58  $\mu$  per second. This speed is modified by the action of temperature and other factors in the environment. Defective oxygen does not stop the movement, but slows it. The presence of calcium is necessary for this type of locomotion. If an ameba is placed in the presence of a substance which extracts calcium (such as an oxalate) the motion is stopped (Pantin).

The potassium ion has an action antagonistic to that of the calcium ion. A severe mechanical injury, an electrical shock, or the action of ultraviolet radiation, will call forth a rigid retraction of the pseudopodia and the cell will acquire a spheroidal form.

The analysis of the physicochemical conditions which modify amoeboid motion, the use of micromanipulation and particularly studies in colloidal chemistry of protoplasm have forwarded interpretation of the mechanism of this motion. Early theories attempted to explain it on the basis of the general contractility of protoplasm. Then for some time it was supposed that the formation of the pseudopodia was due essentially to a localized decrease in the surface tension of the cytoplasm at the interphase. This theory was based particularly on model experiments, some of them carried out with drops of oil or of mercury which, when subjected to the influence of agents which lower surface tension at a certain point, assumed forms similar to those seen during amoeboid motion.

At the present time the explanation based upon physicochemical modifications of the protoplasmic colloid (solation-gelation) acquires more importance. The formation of pseudopodia is supposed to be due to a localized superficial liquefaction, followed by a flowing of a current of endoplasm into its interior (Rhumbert).

It is interesting to recall that in the course of cell division similar phenomena occur. There is a temporary dedifferentiation of the cell with a loss of its structural characteristics and at the same time an emission of small pseudopodia from its surface, just as in amoeboid motion.

The causes of this thixotropic change are not explained. Some authors have supported the idea that the solation occurring at the point of the pseudopod is due to an acidification of the medium, but it has not been possible to confirm this. It also has been supposed that the plasmagel of the amoeba is liquefied by agents which extract calcium (Heilbrunn). In the amoebocytes of the invertebrates it has been found that in the thixotropic transformation which characterizes amoeboid motion, sulfhydryl groups (SH) are liberated and will give the positive Hopkins nitroprusside reaction. As this occurs also in the course of protein denaturation, it has been suggested that a similar phenomenon takes place in amoeboid activity (Farré-Frémont). This partial denaturation would bring with it a change in the bonding between the colloidal micelles and the dispersing water and, therefore, a liquefaction of the protoplasmic gel.

Present theories of amoeboid movement are based on the modern knowledge of submicroscopic structure of protoplasm (see Chapter IV) and particularly on the consideration that there is in it a network of polypeptide chains held together by different

kinds of cross linkages (Van der Waal s forces, hydrogen bonds or stronger bonds) Changes in such forces and in the degree of folding or length of the chains may cause sol  $\rightleftharpoons$  gel transformations in a localized region of the protoplasm In such a system protoplasmic contraction might occur either by a rearrangement of the side-chain connections resulting in a narrowing of the meshes of the molecular reticulum, or by a folding of the protein chains" (De Bryun)

Another important factor in ameboid motion is *adhesion to a solid support* An ameba which floats freely in the liquid medium can emit pseudopodia, but does not progress only when it adheres to a solid surface does it commence this type of locomotion In tissue cultures the fibers of the coagulum serve for the support of the ameboid cells in connective tissue the collagenous or reticular fibers serve this purpose

Substances which influence the motion by attracting or repelling the cells should be placed among the factors determining ameboid motion This property which is called chemotaxis, has great importance in the defense mechanisms of the organism and especially in inflammation

### Ciliary Movement

In contrast to ameboid movement which takes place upon a solid substrate and involves a deformation or change in shape of the entire protoplast, ciliary movement is adapted to a liquid medium and is executed by minute specially differentiated appendices These are contractile filaments, variable in size and number called *flagella* if they are few and long cilia if short and numerous Between the two extremes there are intermediate forms but in all cases the mode of implantation and the essential characteristics are similar

These motile prolongations are relatively common throughout the zoological scale and also are found in some plant cells In the Protozoa, and especially in the Infusoria there are hundreds or thousands of minute cilia for each cell and their movement permits a rapid rotation and progression of the animal in the liquid medium In some special regions of these Infusoria various cilia fuse and form larger conical appendices the *cirri* or membranes known as *undulating membranes* Although the morphology of these appendices is somewhat different, the functional significance is similar to that of the cilia and flagella

Many bacteria have flagella In Protozoa one entire class, the Flagellata, is characterized by the presence of these appendices Among the Metazoa the spermatozoa have the property of pro-

gression as isolated cells using flagella. On the other hand, it is relatively common to find epithelial cells which possess vibratile cilia and constitute true ciliated sheets. These at times cover large areas of the external surface of the body and determine the motion of the entire animal. Such is the case with some of the *Platyhelminthes* and *Nemertinea* and also with the larvae of the *Echinodermata*, *Mollusca* and *Annelida*. More often the ciliated epithelial sheets line cavities or internal tubes of the multi-cellular animals, such as the air passages of the respiratory system or various parts of the genital tract. In these organs the movement



Fig. 121 Cylindrical epithelial cells with vibratile cilia from the human oviduct. Iron hematoxylin.

of all of the cilia is orientated in a single direction, thus causing fluid currents destined to eliminate, as seen in the respiratory system, solid particles in suspension which might cause damage to the organism, or to move along immobile cells. A good example is that of the egg of the amphibia or of mammals which traverses the whole course of the oviduct driven with the aid of vibratile cilia. In some epithelial cells there is a single central flagellum in connection with the cell center. A typical ciliated cell is generally prismatic and the cilia are implanted along the free surface. In fixed and stained sections they appear as short hairlike appendices, the implantation of which in the cell is relatively complex. In general, each cilium passes across a thick cuticle, which is a differentiation of the cell membrane, and ends in a minute granule called the basal granule (Fig. 121). In some cases,

coming out from the basal granule and situated in a row beneath the cuticle, there arise fine fibrillae, the *roots of the cilia*, which converge into a conical bundle, the pointed end of which terminates at one side of the nucleus

The significance of the basal granules has been the subject of much discussion. They often have been considered as derivatives of the centriole (Chapter V) (theory of Meves Hennegy and Lénhossek) and this seems to be true in some cases, as for example, in the cells which line the kidney tubules of salamanders or the seminal vesicles of man. All of these cells show a long cilium implanted on a true but superficial centriole. Another example is the flagella of the spermatozoa which are derived from the centrosomic apparatus. Nevertheless, it appears certain in other cases that the basal granules have no relation whatever to the cell center. In the epithelia of the invertebrates the vibratile cilia may lack basal granules.

In various epithelia there are appendices similar in form to cilia, but immobile, and called *stereocilia*. Examples are the prolongations of the epithelial cells of the epididymis which seem to intervene in the elimination of the cellular secretion, or the rigid filaments of the cells of the macula and crista of the internal ear which serve as receptors of stimuli and transmit them to the cells.

Cilia and flagella are extremely delicate filaments whose thickness is often at the limit of the resolving power of the microscope and, for this reason, they generally show no internal structure. Some flagella nevertheless, as in the case of the tails of the spermatozoa have an axial filament and a peripheral sheath.

Recently a considerable advance has been made in the study of the ultrastructure of the cilia and flagella. The most important result of these investigations has been the assigning to them of a structure somewhat similar to that of muscle fibrillae. With polarization optics (Chapter IV) the cilia and the flagella show a positive intrinsic and form birefringence, which leads to the belief that they are composed of submicroscopic fibrillae orientated along the length of the axis (W. J. Schmidt). Direct evidences of this ultrastructure have been obtained with the electron microscope. In the tails of spermatozoa, in flagella and in vibratile cilia, submicroscopic fibrillae are found which are uniform in number for each case and between 250 and 600 Å thick. In the normal state these fibrillae are firmly bound together to form bundles (Schmitt, Hall and Jakus) (Fig. 123). It is an interesting fact that in tails of the spermatozoa the number of fibrillae is relatively constant, varying between nine and twelve. In the spermatozoa of mammals

the bundle of fibrillae is surrounded by a sheath which contains a helical fibrilla

An analysis of ciliary movement can be made easily by scraping with a spatula the epithelium of the pharynx of the frog or toad and placing the scrapings in a drop of physiological salt solution, between a slide and coverglass. On the free surface of the epithelial cell the vibratile cilia will be seen in very rapid motion. If a row of cilia which are in process of contraction is observed it is seen that the contraction is metachronic in the plane of the direction of motion, that is, it is initiated before or after that in the next cilium, in such a manner that in the over all picture true waves of contraction are formed. (Fig. 122)

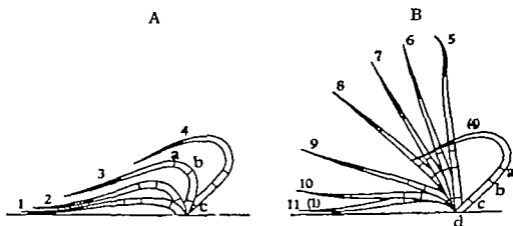


Fig. 122. Diagram of ciliary movement. To facilitate comparison, the various regions of the cilia have been marked with letters. The different stages in movement are indicated by numerals. (From Kinoshita and Kamada.)

On the other hand, in a row perpendicular to the direction of motion, the contraction is isochronic: all of the cilia are found in the same phase of contraction at a given time. This coordination of ciliary movement implies the existence of a mechanism of regulation, the nature of which is unknown. This mechanism of regulation evidently does not depend upon the nervous system, since it persists even after the separation of the epithelium from the rest of the organism. However, for its maintenance the existence of a cytoplasmic continuity is indispensable, since if a cut is made in the row of cilia the waves of contraction of the two isolated pieces do not coordinate their movements. The direction of the effective ciliary beat appears to be a fixed characteristic which depends also upon the underlying cytoplasm. If a piece of epithelium is removed from the pharynx of a frog and is implanted with reversed orientation, the movement is retained but in a direction opposite to that on the remaining intact epithelium.

The contractions of the cilia are generally very rapid (10 to

17 per second in the pharynx of the frog) for which reason they are difficult to follow under the microscope. Analysis of the movement has been facilitated greatly by ultrarapid cinematomicrography which permits one to follow the various phases in the contraction of a cilium and to calculate the duration of each cycle (Fig 122)

Ciliary movement presents varying characteristics in different cells and, in general, may be pendulous, unciform (hooklike), infundibuliform, or undulant. The first two are carried out in a single plane. In the pendulous movement, typical of the ciliated Protozoa, the cilium is rigid and the motion is carried out by a flexion at its base; on the other hand, in the unciform movement, the most common type in the Metazoa, the cilium upon contraction is doubled and takes on the form of a hook. In the infundibuliform movement, the cilium or flagellum rotates, passing through three mutually perpendicular planes in space describing a conical or funnel-shaped figure. In the undulant motion, characteristic of the flagella and membranes, there are waves of contraction which proceed from the site of implantation and pass to the free border.

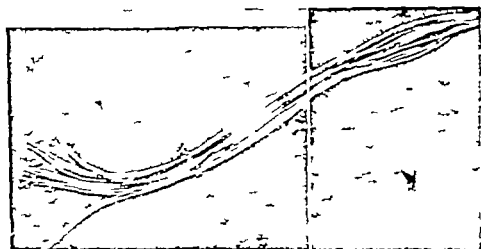


Fig 123 Electron micrograph of a cilium from *Paramecium* in shadow-cast with chromium  $\times 11,000$  (Courtesy of M. Jakus and C. E. Hall, and of *Biological Bulletin* 19 ( ) 131 1946)

The intimate mechanism of ciliary contraction is not known although various theories have been advanced for its interpretation. One of the oldest of these proposes the existence of fluid currents which pass alternately from the cell to the cilium and vice versa. According to this theory, cilia as well as flagella are regarded as hollow tubes in which the cell fluid could flow in a rhythmical manner. A more recent theory also explains the mechanism of ciliary movements on the basis of changes in water content, but with a different physicochemical foundation. The two sides of a cilium are believed to differ in their capacity to absorb water in such a way that the increase in the number of water molecules on one side would cause the cilium to curve toward the opposite side (Gray)

This theory does not interpret satisfactorily the cases of reversibility of the ciliary beat which, although rare, do exist in nature. This phenomenon implies that such a difference in hydrophilia could be reversed.

Modern studies on the ultrastructure of the cilia and flagella, by demonstrating in them the existence of submicroscopic fibrillae arranged in bundles, place the interpretation of the mechanism of the ciliary beat on a completely different basis, since the motion toward one side or the other would depend upon unequal contraction of the submicroscopic fibrillae. On the other hand, these conclusions tend to homologize the structure of the cilia and flagella with that of the myofibrillae of the muscle cells, which would lead us to suppose that the mechanism of contraction is similar in the two cases. The consideration of the modern theories of muscular contraction is beyond the scope of the book, we shall say only that they are based on the submicroscopic configuration of the micelles of actomyosin which make up the myofibrilla, and on the possibility that this configuration changes at each contraction (see Chapter XII)

## VISIBLE MANIFESTATIONS OF THE NUTRITION OF THE CELL

### Phagocytosis

Most cells, free or in tissues, receive their food in a state of solution. In the complex Metazoa substances are digested by enzymes in the interior of the digestive tube and, after absorption, pass to the internal fluids where they possess molecular dimensions. The passage of these molecules of food across the plasma membrane is invisible with the optical microscope and has been discussed in Chapter VI, under the more general subject of cell permeability. In some cases, nevertheless, cells may actively ingest rather large, solid particles, and then the process of penetration generally is visible with the microscope.

This latter activity which is called phagocytosis (Gr. *Phagcin* to eat) is found in a great number of Protozoa and among certain cells of the Metazoa but, in the latter rather than being a phenomenon of nutrition, it is, in general, a means of defense which permits the ingestion of bodies which are foreign to the organism, such as bacteria, dust particles and various colloids. In some cases phagocytosis serves also for the reabsorption of portions of an organism which are in the process of degeneration (for example, in the process of disappearance of the tail of the larvae of amphibia in the course of metamorphosis, or in the

destruction of cellular elements which must be continually renewed, as is the case with the red blood corpuscles in the spleen).

Among the mammals this property of phagocytosis is found very highly developed in the granular leucocytes and also in the cells of mesoblastic origin which ordinarily are grouped under the common term of the *macrophagic system* (Metschnikoff) or *reticulo-endothelial system* (Aschoff). The cells belonging to this group include the histiocytes of the connective tissue, the reticular cells of the hematopoietic organs (bone marrow, lymph nodes, spleen) and those endothelial cells which line the capillary sinuoids of the liver, the adrenal gland and the hypophysis. All of these cells can ingest not only bacteria, protozoa and cell debris, but also smaller particles of colloidal nature. In the latter case, phagocytosis receives the name of *ultraphagocytosis* or *colloidopexy*. When the absorbed colloid is a chromogen the property receives also the name *chromopexy*. An example of chromopexy is the capacity which the mesoblastic cells have to ingest and store vital dyes of a colloidal nature.

These various aspects of phagocytosis which include the ingestion of particles of widely varying sizes are distinct from the phenomena of permeability by means of which the cells absorb substances in solution.

Among the Protozoa phagocytosis is intimately linked to ameboid motion. The amebae ingest large particles, including micro-organisms, surrounding them with their pseudopodia to form a food vacuole within which the digestion of the food takes place later. Nevertheless, small particles may be ingested without the formation of vacuoles. In the leucocytes and other phagocytic cells of multicellular animals the act of phagocytosis in some cases is not linked to ameboid motion and may be carried out even by completely immobile cells.

Phagocytosis has a great importance in pathology as a general mechanism for defense of the organism, and this aspect is dealt with extensively in textbooks on microbiology and general pathology. Here we shall refer more particularly to the cytological and physicochemical aspects of this function of the cell.

In analyzing the process of phagocytosis one may distinguish two distinct phenomena. The first is that of *adhesion* or *adsorption* of the particle to the mass of the protoplasm and the second is the penetration proper of the particle into the interior of the cell. In some cases it has been possible to dissociate these two phases of phagocytosis. Thus at low temperature bacteria may adhere to the cytoplasm of the leucocytes without being ingested. The phase of adsorption which is comparable to a

process of agglutination, seems to obey physicochemical factors, such as electrostatic charges on the surface, but it is also likely that phenomena of chemical affinity may take part in it. The ingestion of the particle may be considered as the result of the extension of the superficial cytoplasm or ectoplasm upon the interface (Fauré-Fremiet). The macrophages, for example, put out hyaline lamellar pseudopodia which adhere to and extend over the surface of the particle until it is completely surrounded. These pseudopodia usually are very delicate and generally reach a thickness of only  $0.25 \mu$ . In the extension of these prolongations, surface tension forces and a certain affinity between the protoplasm and the particle intervene, comparable to what occurs when a liquid wets and extends over a solid surface. In Chapter III we mentioned the important role which the forces of surface tension may play in the experimental penetration of lipid droplets into the cytoplasm.

In ultraphagocytosis or ingestion of particles of submicroscopic dimensions similar phenomena intervene but others, of a more complex nature, may also take part here. Among them are the electrical charge of the particles, the degree of dispersion of the colloid and the manner of introduction. Macrophages accumulate the acid vital dyes, such as pyrrole blue, trypan blue and lithium carmine, which have negative charges, and other negatively charged colloidal substances such as colloidal silver, iron saccharate and india ink (Schuleman and Evans). The degree of dispersion of the substance, which depends upon the size of the particle (v Möllendorff) also plays an important role. The vital acid dyes have very small particle size and consequently a great power of diffusion. Nevertheless, in order to be ultraphagocytized, they must be previously attached to a protein, which acts as a vector. If an animal is injected with a vital dye of the type mentioned above, or with a negatively charged colloid, all of the cells of the macrophagic system accumulate it in a progressive manner. If the process is followed in vivo it is seen that these substances are deposited at first in the form of very small granules which progressively increase in size until they constitute true intracellular precipitates. The fundamental characteristic of this system is that it accumulates and concentrates these dyes and colloids, even when they are administered in very dilute solutions. With massive injections, other cells not belonging to this system can also ingest such substances. In Chapter V we mentioned the relation which exists between the Golgi apparatus and the accumulation of these ultraphagocytized substances.

**Inclusions or Paraplast (Gr *Para* at one side)**

When foodstuffs penetrate in solid form (phagocytosis) they may be digested by intracellular enzymes until they reach a state of solution. Under these conditions they are generally used by the protoplast as a source of energy or in the synthesis of its own substance, but in certain cases they may accumulate and constitute parts visible by cytological and histochemical methods. These reserve substances are grouped under the generic term of *cellular inclusions* or *paraplast*. They do not have a composition identical



Fig. 124. Goblet cell from the intestine of *Amblystoma*. The other cells show a very definite striated border. Iron hematoxylin.

to that of the food ingested by the cells, since they undergo a certain degree of transformation. Nevertheless, they should be considered as a relatively inert material. Examples of this type of paraplast are glycogen and the droplets of fat in animal cells.

In other cases paraplast is a product of cell activity which accumulates in the cell to be excreted to the outside later or to be used by other cells. Such, for example, are the droplets of mucinogen of the muciparous cell (Fig. 124) or the secretion granules of the secretory cells (Fig. 125).

Finally, many processes in the catabolism of the cell may manifest themselves in the accumulation of inert substances such as crystals and pigments which in many cases are signs of a state of senility or of degeneration of the cell.

The analysis of the nature and significance of all of the cell products which may be considered as paraplast lies beyond the limits of this book, we shall refer here only to some common characteristics of all of them and shall mention the most important of such substances in animal cells

From the *cytomorphological* point of view, paraplast can assume extremely varied forms and localizations. In some cases it may be a diffuse homogeneous substance in the cytoplasm, demonstrable only by histochemical methods (Fig 16). In others, there are minute cavities called *vacuoles* which have contents



Fig. 125 Cells of the pancreatic acini. The apical part of the cells is occupied by numerous granules of zymogen. In the base are the nuclei with nucleoli and, in the cytoplasm, elongated chondriosomes. Fixation: Champy; Stain: Altmann's fuchsin.

more liquid than the rest of the cytoplasm and which are separated from the latter by a membrane with characteristics of structure and permeability similar to the plasma membrane. Such vacuoles are very common in plant cells and also in many Protozoa. In animal cells they are relatively rare. Another type of paraplast is represented by the *droplets* of fat which have a spherical form and are sharply delimited from the rest of the cytoplasm with which they are not miscible (Fig 38). These droplets generally are liquid at body temperature. There may also be accumulations of a spherical form and with a consistency greater than that of the remaining cytoplasm. Such are the granules of zymogen of the cells of serous secretion (Fig 125), the granules of mucinogen of the muciparous cells (Fig 124) or the granules of pigment. Finally, there may be inclusions which are completely solid, either amorphous or crystalline. This type is rare in

animal cells but much more common in plant cells (such as calcium oxalate)

The *chemical composition* of the paraplast is extremely variable and predominating substances may be proteins, fats, carbohydrates, or minerals

*Protein inclusions* are relatively rare, as usually these substances are incorporated into the protoplasmic mass. This lack of separate accumulations of proteins in the cells raises the problem of the reserves of such substances in the organism. As we shall see later there are numerous reserves of carbohydrates and of fats in the various tissues in the form of paraplast which can be utilized when necessary. Much of the reserve protein is contained in the protoplasm itself. When there is a dietary deficiency proteins are extracted from various tissues and especially from the skeletal muscles which function as true reservoirs of proteins. Nevertheless, in the hepatic cells, granules or irregular flakes of a substance of protein nature may be observed. Yolk bodies also contain proteins. In rare cases there may be observed crystals of protein nature in the cytoplasm or in the nucleus. Furthermore, many secretion granules, such as zymogen (Figs 61 and 63) are of protein nature.

Among the proteins which may constitute cell inclusions the mucoproteins have considerable histochemical interest. These are protein complexes which yield upon hydrolysis a protein, a hexosamine polysaccharide and, in many cases, sulfuric acid thus being made up of a sulfuric ester of polysaccharide. It has been found nevertheless, that in some tissues such as the vitreous body and also in the synovial fluid, the sulfuric acid is lacking (Meyer). Histochemically these compounds show intense basophilia demonstrable even in very dilute solutions and metachromasia which is evident with thiazine dyes such as toluidine blue and methylene blue. In the cells where inclusions of mucoproteins occur as for example those of mucous secretion, the basophilic leucocytes and the mast cells such inclusions, instead of being stained by the ordinary color of the dye, assume a red violet tint (metachromatic reaction). There are also other substances, of an intercellular nature such as the matrix of cartilage, tendons and the cornea, and the gelatinous substance of the umbilical cord which take a similar stain.

This reaction is so selective that it can be observed even with a dilution of the stain of 1:200,000. In greater dilutions the compounds which contain mucosin-sulfuric acid (such as the cells of mucous secretion) are not stained while those which have chondroitin-sulfuric acid (cartilage connective tissue) show an

intense affinity (Hempelmann) According to Michaelis and Granick, metachromasia depends on the formation of dimeric and polymeric molecular aggregates of dye upon these compounds. The same basic dyes when acting upon nucleic acid do not form polymers In this case each cation of the dye combines with one acidic side chain of the nucleic acid to form a stoichiometrically well defined saltlike compound.

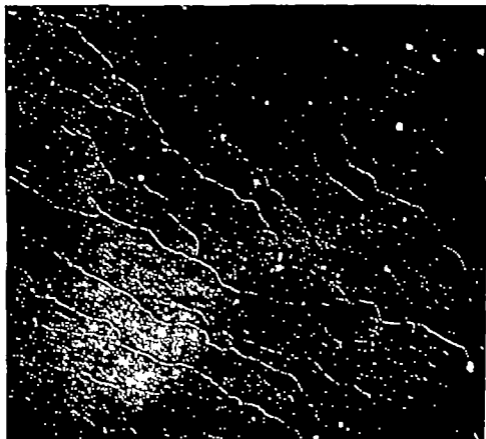


Fig. 126. Electron micrograph of sodium hyaluronate shadowed with chromium (description in the text)  $\times 25,000$ . (Courtesy of J. Gross)

The mucoproteins of the epithelial cells are generally very labile compounds which easily swell and dissolve in water. The best method to preserve them is the freezing-drying technique with which the mucinogen granules can be seen. With chemical fixation the mucinogen granules usually swell and coalesce to form what is called mucus. By means of a mild hydrolysis of the mucoproteins they may be caused to liberate the carbohydrate fraction, which gives a positive Schiff's reaction for aldehydes. These and other tests have been utilized histochemically to characterize the mucoproteins (see Dempsey and Wislocki).

Lately considerable importance has been attributed to the study of acid polysaccharides, particularly hyaluronic acid and

chondroitin sulfuric acid, which are found in the ground substance of connective tissue. The molecular weight of hyaluronic acid is estimated to range between 200 000 and 500 000. In solution it produces a very viscous gel even at low concentrations and shows positive double refraction of flow. Electron microscope studies of hyaluronic acid show long branching and anastomosing fibrous processes of 50 to 200 Å in width (Fig. 126). This compound is readily depolymerized by an enzyme, hyaluronidase, resulting in a rapid drop in viscosity and streaming birefringence of the solution and in marked increase in the permeability of connective tissue in the living animal (Duran Reynals). These phenomena are apparently involved in bacterial invasiveness in the fertilization of the egg and in other physiological and pathological processes.

*Fat inclusions* are frequently found in various types of cells (Figs. 12 and 38). The major reserve for the organism is represented by the adipose tissue, the cells of which can accumulate large quantities of lipids and especially of neutral fats. A large central droplet is formed which pushes the nucleus and the cytoplasm far out toward the cell membrane. The cells of the adrenal cortex also contain considerable quantities of lipids, but in this case in the form of numerous small droplets which contain complex mixtures of sterols, fatty acids and phospholipids.

Droplets of fat can be demonstrated by means of *osmic acid* which stains them black. This reaction is not specific. Staining with Sudan III or scarlet red has a greater histochemical value. These stains act by a simple process of diffusion and solubility and in this way are accumulated in the interior of the lipid droplets. Recently the use of Sudan black B, another stain of the same series, has been initiated. This stain has the advantage of being dissolved in lipids such as the phospholipids and cholesterol, and of producing the greater contrast of these structures (Balcer).

Absorption of fats by the cells of the intestine and perhaps by other cells of the organism implies a previous process of saponification by which the fatty acid is separated from the glycerol residue (Chapter II). These two parts of the lipid molecule are absorbed separately and then are combined within the cell under the influence of lipases. Intracellular synthesis of fat can be demonstrated by histochemical means. If a rat is caused to ingest a certain quantity of olive oil, at the end of thirty minutes the number of droplets of neutral fat, demonstrable by staining with Sudan III, is small. On the other hand, if one uses the method of Fischler, which consists in treating the cells with cupric acetate which forms copper soaps with fatty acids, and then staining with hematoxylin it can be demonstrated that considerable quantities of fatty acids exist at this time (Jekel). Some hours later there can be seen by this method it is known that there is then only a small quantity of fatty acids and abundant droplets of neutral fat.

The presence of the fat droplets in the intestinal cells is only transitory; their

contents soon passing partly to the blood and, in larger quantities, to the lymph of the intestinal villi, to be distributed to the rest of the organism. The passage from the base of the cell to the capillaries is invisible with the microscope and it is supposed that there may be another process of splitting into fatty acids and glycerol (under the action of lipases). Once in the blood stream, the neutral fat is reconstructed and may be seen under the darkfield microscope as small refractile bodies called *chylomicrons*.

The accumulation of fat in any cell begins with the appearance of very fine droplets which then increase in size by apposition and confluence. In Chapter V we mentioned the role which the chondriome appears to play in certain cells in the deposit of fatty droplets.

From the physicochemical point of view the intracellular fat is found in the form of an emulsion stabilized by proteins. This emulsion may be so fine (colloidal) that it is invisible with the light microscope, then constituting a part of the so-called "masked" fat (Chapter II). In other cases the fat constitutes the dispersed phase of the system, the dispersing phase of which is a solution of proteins. Finally, when the lipid content is above 25 per cent, the fat constitutes the dispersing medium and the protein micelles the dispersed phase. Under the influence of a toxic or of other pathological condition, the equilibrium between these two phases may be altered and give rise to morphologically visible changes. Such is the case in *fatty degeneration* or *lipophanerosis* (Gr. *Phaneros* visible) in which there occurs a true "unmasking" of the fat.

*Glycogen inclusions* represent practically all of the carbohydrate reserves of an animal. Since this substance is, in general, soluble and relatively diffusible, it is displaced or precipitated by the common chemical fixatives. This fact gives rise to numerous artifacts of fixation (Fig. 15). The freezing-drying method (Chapter III) permits us to obtain a real image of the distribution of glycogen in the interior of the cells. In the hepatic cells it is generally described as irregular flakes preferentially localized in certain parts of the cytoplasm. However, with the freezing-drying technique it appears diffuse and occupies all of the ground cytoplasm (Fig. 16). It also is diffuse in the cells of vaginal epithelium, in cartilage (Fig. 127) in endometrium and in adipose cells (Fig. 128). In the leucocytes and in muscle fibers it appears in the form of fine granules (Fig. 128). This technique of fixation permits a more intense histochemical reaction and a greater sensitivity to tests with glycogenolytic enzymes; it also impedes the partial autolysis of glycogen and preserves its chemical properties (Mancini).

Recently it has been possible to demonstrate that the diffuse

glycogen of the hepatic cells is composed of submicroscopic particles (LERNER). These particles were isolated from a brei of liver tissue, previously centrifuged to the point where all of the visible components (cell debris, nuclei, chondriome) had been deposited, and then ultracentrifuged at 12 000 r p m. The sediment thus obtained shows ultramicroscopic particles (demonstrable by darkfield examination) constituted of glycogen, protein, and 75 per cent water. It is interesting that the protein represents only about 1 per cent of the dry weight. These studies are interpreted as indicating that the hepatic cell polymerizes glucose into glycogen, thus separating it immediately from solution and



Fig. 17. *Left* Glycogen in cartilage cells. Chemical fixation, iodine reaction. The distribution in flakes is seen. *Right* Glycogen in cells of cartilage fixed by the freezing drying method. Iodine reaction. The glycogen has a diffuse and homogeneous distribution in the cytoplasm. (Courtesy of R. E. Mancini.)

fixing it to proteins which constitute the submicroscopic particles. It is possible that the glycogen of muscle and of leucocytes, which forms microscopic granules, may contain a greater amount of proteins and, for this reason, show more stability. Nevertheless it is also possible that these differences are due to a different degree of polymerization. The poorly soluble glycogen (*desmoglycogen* of Willstätter and Rohdewald) may differ from the more soluble (*lyoglycogen*) by being composed of polymers of greater molecular weight (Meyer).

Histochemical study of glycogen can be carried out with the iodine reaction, which gives a characteristic mahogany color or with Bauer's reaction in which, after treatment with chromic acid, glycogen is colored by Schiff's reagent (leucofuchsin). Best's carmine method, although it gives good images, is not specific.

All of the methods require a control by means of ptyalin, which digests the glycogen, followed by the appropriate histochemical reaction

Recently advances have been made in the technique of the iodine reaction. The improved method applies the iodine in a nonpolar solvent to the frozen and dried sections and provides for increased permanence and stability of the preparation (Fig 128). In this way, the demonstration of glycogen can be carried

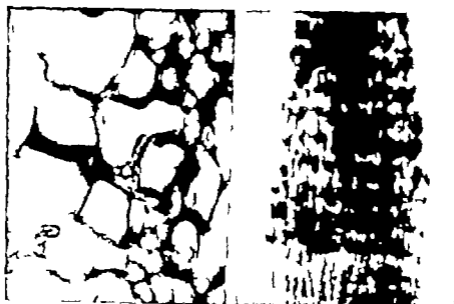


Fig. 128. *Left* Glycogen in adipose cells. The freezing-drying method permits the demonstration of the presence of glycogen diffusely distributed in the cytoplasm surrounding the large droplets of fat. Fixation, freezing drying, without denaturation. Iodine reaction. *Right*. Granular glycogen in a striated muscle fiber. The minute granules are arranged in a double row and are uniform in the different sarcomeres. Technique as for the adipose tissue. (Courtesy of R. E. Mancini.)

out under ideal conditions, without appreciable displacement or loss of this material (Mancini)

By means of histochemical studies an attempt has been made to correlate the distribution of glycogen with the localization of the phosphatases. This study offers considerable interest because, from the biochemical point of view it is well known that the synthesis of glycogen requires the intervention of enzymes, such as alkaline phosphatase, which are capable of liberating the phosphate from the phosphorylated intermediaries involved. This would lead us to suppose a topographic relation between the glycogen and the alkaline phosphatases, as they are demonstrated by the technique of Gomori. Recent studies on the maternal and on the fetal placenta of various species of animals (Dampsey and Wislocki) tend to support this hypothesis

*Pigmentary inclusions* are substances of a heterogeneous nature which have in common the property of being colored materials (chromogens). The pigments are generally a product of cell metabolism (endogenous pigments) but, in many cases, they are derived from the outside or from other tissues and are accumulated by the cells (exogenous pigments).

Among the exogenous pigments some important ones of plant origin should be noted, such as the *carotenoids* among which are the carotenes, xanthophyll and lutein. On page 25 we studied the general chemical composition of those pigments which result from the polymerization of the unsaturated hydrocarbon, isoprene. *Carotene* is accumulated particularly in the hepatic cells and is transformed into vitamin A. *Lutein* is the pigment which colors the yolk of the egg. All of these carotenoids are characterized by great solubility in the fatty substances to which they impart their own color.

To this group of exogenous pigments belong also the *blood pigments* which come from the hemoglobin of the blood or from the myohemoglobin. The most important of these are (1) *Hemosiderin* which results from the disintegration of the red corpuscles in the reticular cells of the spleen and in other macrophagic elements of the organism. This pigment, which has a brown color and appears as an amorphous granular substance is characterized by the presence of iron in the ionic state which can be demonstrated easily by the appropriate histochemical reaction. (2) *Hematoidin* a crystalline pigment which lacks iron and is generally found in tissues where extravasation of blood has occurred. The latter pigment is similar in its composition to the *bilirubin* secreted by the liver.

Among the pigments of endogenous origin the most important are the *lipochromes* and the *melanins*. The former are derived from the lipids and perhaps from the phosphatides, by a transformation which is poorly understood and are found in the cells in the form of granules of a yellow or brown color which show characteristic properties of lipids.

*Melanins* are pigments which contribute to the color of the epidermis, hair and other portions of the skin and which are localized particularly in epithelial elements and in some of the connective tissue cells (melanophores). They appear in the form of granules of a brown or black color and are very resistant to reagents. Their origin has been much discussed. It is generally considered that they are phenolic polymers which may be derived from the oxidation and the condensation of the products of protein catabolism but the nature of the *melanogenic* substance

is not known exactly. According to some authors, it is formed from tyrosine, which is oxidized by an enzyme, *tyrosinase*. Others consider that the adrenalin, produced by the medulla of the adrenal gland, may be the mother substance of melanin. Still others assign special importance to dihydroxyphenylalanine which can be oxidized by a *dopa-oxidase* (Bloch). The *lipofuscins* are at the present time considered similar to the melanins. They are brown pigments, also called pigments of 'exhaustion' because they are found in various organs (heart, muscle) in a state of *brown atrophy* or in the nerve cells of senile individuals.

## CYTOMORPHOLOGICAL ASPECTS OF CELLULAR SECRETION

### Secretory Cycle

*Secretion* is the process by which cells absorb substances, transform them chemically or in concentration, and expel them. The products of secretion may be utilized by other cells, may stimulate or inhibit other cells, may act chemically on other substances or may be eliminated from the organism. These transformations imply work done by the cells, since, in chemical transformation or in fluid transfer against a concentration gradient, a certain quantity of energy is always consumed. In *excretion* nonmodified substances are expelled along favorable concentration gradients without expenditure of energy by the cell. Nevertheless, both processes frequently are more or less intermingled and it is difficult to separate them clearly.

The secretory cell may be compared to a complex machine the functioning of which leads to the elaboration and the elimination of the products of secretion. This process implies the existence of a continuous change in cellular activity which can be interpreted only when it is studied in all its different stages. If fixed and stained secretory cells are studied under the microscope, the image which is obtained is the representation only of a single stage of cell work. In cell secretion, more than in any other process, the factor of time must be taken into account in order to interpret the results of cytomorphological analysis. For this reason the best technique to study the process of secretion is vital observation (biomicroscopy) over a sufficiently long period of time.

However this is not always feasible and often offers great difficulties. Another obstacle is due to the fact that in a single gland the cells are generally found in various phases of secretory activity in such a way that in the histological section they present very different morphological aspects. This obstacle may nevertheless be surmounted by using appropriate stimuli which rapidly

modify the activity of the cells (which normally would be functioning in an asynchronous or semisynchronous manner) and drive them in a given direction, thus establishing functional synchronization. If for example, one desires to study the secretion of the exocrine pancreatic cells, the animal is first made to fast in order to bring about a state of repose of the gland, and then the cells are stimulated by pilocarpine, the action of which brings about the rapid excretion of the products of secretion. In this way the various phases of cellular activity are synchronized and practically all

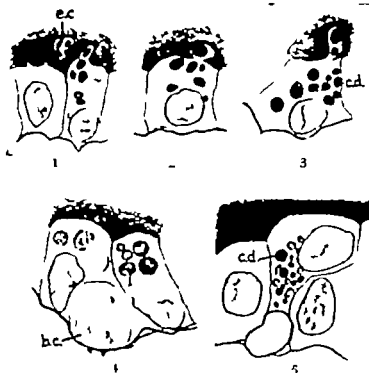


Fig. 109. Process of secretion in the thyroid gland, studied by the freezing-drying technique. 1 and 3. Thyroid cells, 30 to 60 minutes after the injection of the thyrotropic hormone of the hypophysis. The formation of droplets of colloid and their secretion into the cavity of the follicle are seen. *ec*, excreted droplet. *cd*, colloid droplet. 4. Thyroid cells, three hours after injection. The process of reabsorption of the colloid predominates. *bc*, basal colloid. 5. Thyroid cell 2 hours after injection. The formation of the secretory polaris is seen. Fixation by the freezing-drying method. Stain: niline blue and orange G.

of the cells expel their contents and then recover gradually. The cytological study is carried out at various times after the application of the stimulus and can be done in a purely qualitative or in a quantitative manner applying the statistical method (C. G. Hirsch). In this latter method an attempt is made to find any particular state of the cell (for example mitosis, etc.) and to count the cells in this state to see in what proportion they appear at different times following the stimulus.

The secretory cycle has extremely variable cytological expressions, but it generally is characterized by the appearance of products visible with the microscope which are accumulated in the cell and then are eliminated. These may be dense and refractile granules, vacuoles, droplets, and so on, which almost always occupy a definite position in the cell and which, at times, have characteristic histochemical reactions (Figs 124 125, 129 and 130)



Fig. 130 Secretory process in the adrenal medulla. *Left*, a nonsecreting zone. *Right*, a secreting zone. Adrenalin-containing secretion droplets are present in the secreting zone, the plasma, in the veins, is dark. (Beinnett, H. S.; *Am. J. Anat.*, 69-1941)

In some glands, nevertheless, it is not possible to demonstrate by cytological methods any product of secretion, even when the physiological data indicate secretion is active. A typical example is that of the parathyroid, a gland which secretes a powerful hormone regulating calcium metabolism. However the cells of the parathyroid show no product which might be considered as a presecretion or an intracellular precursor of the secretion.

In these cases the existence of the secretory cycle may be demonstrated by taking into account the modifications which are produced in the nucleus and in the cytoplasmic components when the normal activity of the gland is modified. In the parathyroid, for example, the injection of a single large dose of parathyroid extract brings about a great hypofunction followed by a slow recuperation. Cytologically it is seen that in the first stage the dark, osmophilic cells disappear and all of the cells

acquire a homogeneous cytological aspect with a reticular Golgi apparatus of a simple type (Fig 131 A) In the state of recuperation the cells show a functional asynchronism by the heterogeneity of their cytomorphological aspect. There are certain larger cells with a fragmented and vacuolized Golgi apparatus and

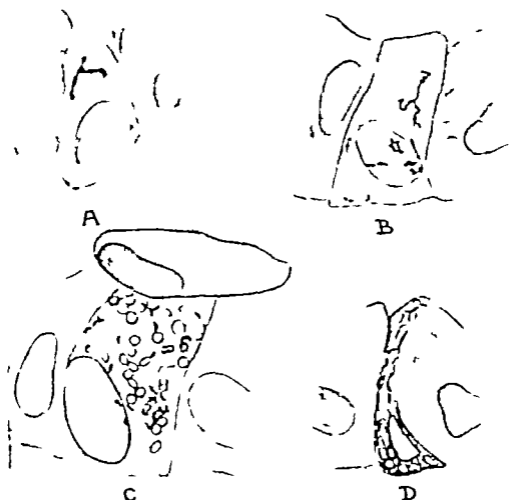


Fig 131 Parathyroid cells in various stages of their secretory activity. A cell in repose with a simple Golgi apparatus. B hypertrophy and fragmentation of the Golgi apparatus. C cell apparently in a state of accumulation of the secretion, vacuoles with osmophilic borders, Golgi apparatus completely fragmented. D dark cell in stage of excretion. Osmic impregnation.

vesicular chondriosomes. The dark, osmophilic elements reappear. In the same gland, the functional hyperactivity, which is obtained by feeding the animals with rachitogenic diets, exaggerates the cytological heterogeneity and increases the number of osmophilic cells. From these experimental studies one can infer approximately what is the normal cytomorphological cycle of secretion even though the product elaborated is not visible (Fig 131).

As an example of a secretory cycle in which the secretion products are very readily visible, we may cite the case of the exocrine pancreatic cells, in which the cycle has been carefully studied (Cowell, Ries, Hirsch). These belong to the group of cells of serous or zymogenic secretion, so-called because they produce a protein secretion rich in enzymes

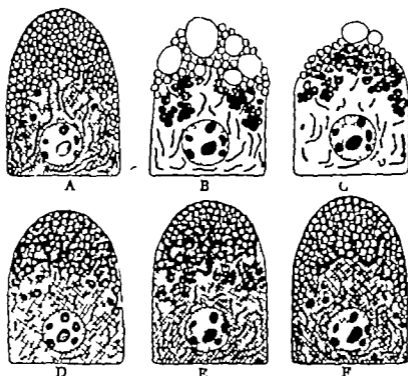


Fig. 132 Secretory cycle of the pancreatic cell of the white mouse. *A* cell from a fasting animal. Zymogen granules and chondrioconts abundant chromidial substance. *B* the same a half hour after injection of pilocarpine. Vacuolization and excretion of the zymogen Golgi apparatus increased in size, disappearance of the chromidial substance. *C* one hour later excretion almost complete. Great osmophilus of the Golgi apparatus. *D* after four hours. Typical Golgi net with newly formed granules. *E*, after seven hours, the process of recovery continuing. *F* after fourteen hours. Recovery completed (After Ries)

The structure of a pancreatic cell has been described elsewhere in this book. In the resting state it presents a typical polarization of its components. The base of the cell is occupied by the nucleus (Fig. 125) the chromidial substance containing ribonucleoproteins (Figs. 63, 64 and 65) and by elongated chondrioconts orientated in the apico-basal direction (Fig. 125). The apical or excretory region is occupied by refractile granules with a high protein concentration (Figs. 61, 63, 125). In the supranuclear zone and among the zymogen granules there is a Golgi apparatus with a reticular appearance.

Injection of pilocarpine brings about a liquefaction of the

zymogen granules and the rapid expulsion of their contents (Fig 132) By means of biomicroscopy it has been seen that, in these cases the passing out of the products of secretion can occur directly through the rupture of the cell membrane and the giving off of parts of the cytoplasm (Cowell) Later the cells elaborate more secretory granules which are accumulated at the apical pole and at the end of several hours regain the same appearance which they had at the beginning (Fig 132) During this stage

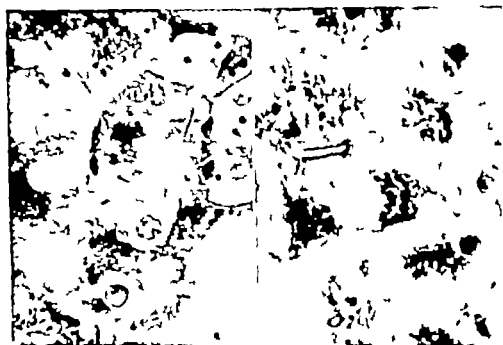


Fig. 133. *Left* Photomicrograph of the exocrine pancreas in a state of repose. The acini are loaded with granules of zymogen the Golgi apparatus is not clearly seen and is situated between the granules Osmic impregnation. *Right*, pancreas activated by the injection of pilocarpine The diminution of the zymogen and the great increase in osmophilia of the Golgi apparatus can be seen Osmic impregnation.

the Golgi apparatus hypertrophies and becomes intensely osmophilic (Fig 133) The chondriosomes seem also to hypertrophy although this is not certain, and the chromidial substance shows a diminution of its ribonucleic acid content (Caspersson and collaborators)

In studying the secretory cycle the method of fixation by freezing and drying (Chapter III) presents great advantages, since it permits us to stop the cellular processes in the most rapid possible manner and thus to determine better its different stages In addition, it permits us to see, under the best conditions and without changes the soluble products and protein secretion when present in high dilution In the case of the thyroid, this method demonstrates an intracellular colloid which is not observable by

other methods and permits us to follow the different stages of its formation and excretion

If an animal is injected with thyrotropic hormone, at the end of a few minutes there appear numerous droplets of colloid at the apical pole of the cell which then are excreted into the interior of the thyroid follicle. The exit of these droplets appears to occur in a manner similar to the secretion of the pancreas, by the evagination of the cytoplasm and the rupture of the cell membrane at certain points (Fig 129, 1, 2 and 3) After this first step of apical excretion, the reabsorption of the follicular colloid begins, and it passes through the cell toward the blood capillaries (Fig 129 4 and 5)

### Origin and Significance of the Secretion Granules

One of the most firmly supported concepts in cytology is that the granules, which are observed in the majority of gland cells, represent the product of secretion. Thus, for example, it is believed that the zymogen granules of the exocrine pancreatic cells contain (although in the inactive form of proenzymes) the various enzymes which are found in the pancreatic juice and that the chief cells of the stomach secrete pepsin and the other enzymes of the gastric juice. In the majority of cases, however this concept is not based on direct cytochemical evidence.

In recent years considerable progress has been made in this direction, permitting us to affirm that at least some products of secretion really are found in the granules or droplets within the cells. As a typical example we may cite the case of the pancreas. If the content of granules in the pancreatic cells is determined quantitatively at various times after stimulation with pilocarpine, and at the same time the concentration of carboxy polypeptidase is analyzed, it is noted that a perfect correlation exists between the two (Fig 134) The number of granules reaches a minimum one hour after the injection of pilocarpine, while the quantity of the enzymes is minimal three hours after the injection. This apparent discrepancy is due to the presence in the excretory ducts of the gland of the secretion expelled by the cells. Later the content of granules and the concentration of the enzyme increase in parallel manner. These results provide strong evidence that carboxypolypeptidase is actually in the zymogen granules.

The significance of the secretion granules in the various cells will be solved as specific histochemical methods are developed for the identification of minute quantities of substances within the cells. Recently it has been possible to identify in a

direct from the thyroglobulin in the interior of the thyroid cells by means of ultraviolet absorption spectrophotometry (page 146) (Gersh and Caspersson)

*The theories concerning the origin of the granules* which have been formed over a long period of time are reflections of the dominant ideas in each period. When histological studies began with imperfect microscopes and without staining of the preparation, the most easily visible structure was the nucleus, and nuclear changes were observed in secretion. As a consequence of these facts, Claude Bernard proposed the theory that the nucleus

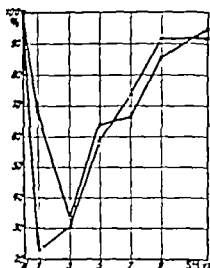


Fig. 134 Content of zymogen granules in the pancreatic cells of the mouse (x x) and content of carbonopol peptidase (o o) expressed in percentage of the values for a fasting animal. On the abscissa: hours after the injection of pilocarpine. (After van Weel and Engel)

elaborates the secretion. This *nuclear theory* was supported even more strongly when nuclear stains began to come into use.

Toward the end of the past century Langley and Heidenhain described the secretory granules and the morphological changes which they undergo during the secretory cycle and suggested that these represent the products of secretion. At the beginning of the present century the *chromidial theory* (Chapter VII) was proposed according to which the chromidial substance comes out from the nucleus and gives origin to the granules.

Later with development of techniques for the demonstration of the chondriome many authors attempted to prove that the granules are derived from the substance of the chondriosomes (*chondriosome theory*). Finally in recent years there has been firm support for the theory proposed by Cajal that the Golgi apparatus intervenes in the formation of the secretion granules.

In Chapter V we discussed this theory in its modern aspects (*theory of the presubstance of Hirsch*)

From this brief review of the theories of secretion which we have made, we can conclude that, with the methods of cytological investigation utilized up to the present, the problem of the origin of the secretion granules cannot be solved. These elements have the characteristic of appearing rapidly in the cytoplasm, passing from the submicroscopic to the microscopic level in such a way that it is very difficult to determine whether they have originated directly or indirectly from the nucleus, from the cytoplasmic organoids, or from the fundamental cytoplasm. In interpreting the intracellular process of secretion, it should be kept well in mind that the cytological changes which are seen in the cell are only an expression of the state of secretory activity. From the microscopic images alone we ordinarily cannot deduce that a causal relation exists between the cytological changes and the elaboration of the secretion, since this latter process is not visible with the light microscope. Nevertheless, recent results support the view that at least in certain secretory cells there is a chemical organization comparable to the morphological organization, particularly in regard to the localization of various enzyme systems. Thus it has been found that the proximal tubules of the kidney where the reabsorption of glucose occurs, are rich in alkaline phosphatase, which is concentrated principally next to the lumen of the tubule in the brush border of the cell. This fact has been related to the processes of phosphorylation of the reabsorbed sugars which take place in the interior of the cell (Gomori). However recently it has also been suggested that phosphatase may act in splitting phosphate esters before these are reabsorbed by the cells (Danielli).

Various observations on the transfer of substances through the cells of the intestine and of the kidney have led to belief in the existence of intracellular systems which would act as carriers or vehicles of these substances. The carrier would act as an absorbent, or as a solvent, or as a chemical substrate for the transported substance. The direction of movement of these carriers from the point where they would be united with the substance to the place where it is eliminated from the cell would be determined in some cases by the structure of the protoplasm. Thus, for example, the long filamentous chondriosomes (batonets) of the renal cells would form true intracellular passageways for the transfer of substances along the long axis of the cells (Höber).

It seems evident that the process of secretion in its intimate details will be better understood when specific cytochemical

methods for the identification and localization of minute quantities of substances within the cells have been developed and when the results obtained by these methods are correlated with the metabolic processes participating in the elaboration of the secretion

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## Chapter VII

# DIFFERENTIATION, SENESCENCE AND DEATH OF THE CELL

### CELLULAR DIFFERENTIATION

In the preceding chapters we have studied the morphological, physiological, physicochemical and cytogenetic aspects of the cell in a general form. Although several diverse types of cells were used as examples (eggs, blastomeres, germ cells, epithelial cells, and so on) in all of them the fundamental characteristics were very similar. We dealt with elements which morphologically are little differentiated (i.e., little specialized). Nevertheless, in higher organisms the multiplicity of functions makes necessary a greater or less degree of specialization and differentiation. Certain cells are adapted to definite functions and, at the same time, their morphology is modified. For example, the nerve cells assume a form and structure adapted to the functions of *irritability* and *conductivity*, thus enabling them to react in a high degree to stimuli and to transmit the signals from one part of the organism to another. Likewise, muscle cells contain *myofibrillae* which have the *property of contractibility* very well developed, although this property is also a general characteristic of living matter.

This progressive specialization in structure and function is what constitutes, in a restricted sense, *cellular differentiation*. Differentiation is always the transformation of something more general and homogeneous into something more specialized and heterogeneous and it is reflected at the same time in both morphological and physiological characteristics.

Cellular differentiation occurs continuously throughout the life of the organism. In the embryonic period it reaches its maximum expression and constitutes one of the most important processes. Every organism develops from a single cell—the fertilized ovum—which has in it the potency to give rise to all of the tissues and organs. This cell divides actively to form the embryonic structure known as the blastula, in which the tissues and organs have not yet been defined. Up to this time in many species this process is mainly quantitative (increase in the number of cells) but after the formation of the blastula it becomes also qualitative. The cells of the blastula then begin to rearrange them-

selves in a process called *gastrulation*, which is a very important step in embryonic development, during which the three germ layers are established and the determination of the future organs is made. At this time and later the process of *cellular differentiation* occurs with the formation of the various tissues (*histogenesis*) and the separation of portions of some of these tissues to constitute the organs (*organogenesis*).

The fundamental processes of embryonic development are, *growth*, *differentiation* and *metabolism*. The first consists in the increase of the spatial dimensions and depends on the multiplication of nuclei or of cells, on the increase in cell size, or on the addition of nonliving substances (paraplasm and intercellular substances). Differentiation is characterized by the increase in complexity and degree of organization and includes *histogenesis* and *organogenesis*. Metabolism is the total of chemical changes which are produced in the embryo and which in part provide the energy necessary for the other processes.

The study of the causes of differentiation lies beyond the limits of cytology but is a special field of embryology, in which one of the most important branches is causal embryology also called the mechanics of development. This science has demonstrated that the cellular differentiation of any region of the gastrula is preceded by *determination*, an invisible process which fixes the destiny corresponding to the future development. This concept is demonstrated in the following experiment (Spemann). If a young gastrula of an amphibian has a portion of its dorsal ectoderm removed (a part which in normal development would be converted into a portion of the nervous system) and if this is implanted into the ventral region of another gastrula, instead of differentiating into nervous tissue, it develops in accord with the region and forms epidermis. If the same experiment is repeated with material from an amphibian gastrula at a later stage of development, the grafted part develops in accord with its site of origin and is transformed into a part of the nervous system. This result demonstrates that at a particular period in gastrulation the destiny of certain parts of the embryo becomes definitely fixed.

The experiments of Spemann and his school have given evidence that all embryonic development, and therefore the differentiation of tissues and organs, results from the coordinated action of organizers, which are special regions of the embryo having the property of determining the differentiation of other regions. Thus, for example, the dorsal lip of the blastopore of the gastrula which is the *primary organizer* determines the

differentiation of the nervous system. In turn, after its own differentiation the forebrain acts as a secondary organizer and determines the formation of the optic vesicle. The latter (tertiary organizer) induces the differentiation of the crystalline lens, which in turn provokes the formation of the cornea. This whole series of integrated processes is thought to depend upon substances (the *evolvers of Needham*) produced by the organizers, capable of bringing about the differentiation of neighboring regions, when these latter are in a state of reactivity or of appropriate *competence*.

If we now confine the problem to its cytological aspects, differentiation may be defined as the process which results in the specialization of the present and potential functions of the cell (Bloom). As it differentiates, the cell adapts itself progressively to a specific function and at the same time loses, in general, the capacity (potency) to carry out other functions. In certain cases differentiation is temporary and reversible and is called *modulation*; in others, it is permanent and irreversible.

It is generally accepted that there is an antagonism between the processes of cell division and differentiation. The latter generally takes place at the interphasic stage, between mitoses, or after cell divisions have definitely ceased. The neuroblasts which originate from undifferentiated cells of the embryonic neural tube and neural crest reach a high degree of differentiation, so that the nerve cell not only loses its capacity to transform into other types of cells but also its capacity to divide. On the other hand, there are in the adult organism other cells which have acquired a lesser degree of differentiation and which under certain conditions can be transformed into different cell types. Such is the case with the reticular cells of the hematopoietic organs which have the power to transform into, and thus to give origin to the cells of the blood or to the various cellular elements of the connective tissue. From these examples it becomes evident that as the cell differentiates its developmental potency becomes restricted. Later we shall see the importance which this has in the senescence of the cell.

### Specific Differentiations of the Cytoplasm

In the course of differentiation the behavior of the nucleus and of the cytoplasm differs markedly. The nucleus never undergoes such definite modifications as the cytoplasm and at times its aspect is very similar in completely different types of cells. It may be said in this regard that just as the nucleus plays a dominant role in *heredity*—conserving intact the undifferentiated material

of the ultrastructure of the myofibrillae of striated muscle (Hall, Jakus and Schmitt) These are composed of a succession of alternating transverse bands which coincide with those observed with the ordinary microscope (Fig 135) The fundamental unit or sarcomere is limited by a narrow and dense band called the telophragma or Z line This is located in the center of the less dense zone known as the I zone, corresponding to the relatively isotropic disc (Fig 137) The A band (which is anisotropic with

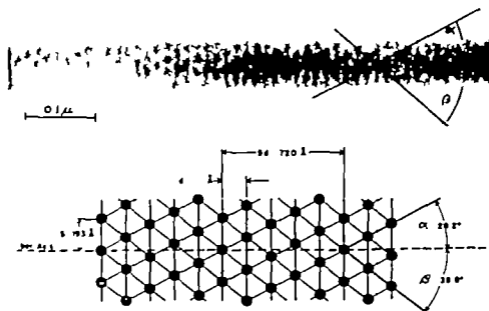


Fig. 136. *Upper* Electron micrograph of a portion of a paramyosin fibrille showing geometrical disposition of regions stained with phosphotungstic acid  $\times 120,000$  *Lower* Diagrammatic lattice showing geometrical relations and dimensions between stained regions in paramyosin fibrillae. (See description in the text.) (Courtesy of C. E. Hall, M. A. Jakus and F. O. Schmitt and of J. App. Phys. 16, 459 1945)

polarized light) has a greater electron density than the I band. Under certain conditions there may be observed in the center of the A band a less dense zone which subdivides this band into two dark semidisks. This zone constitutes the H disc (Hensen's disc) and in its center is the mesophragma or M membrane (Fig 137). The electron microscope, besides revealing these same elements which are observable with the light microscope, shows the existence of longitudinal filaments the width of which is on an average from 120 to 150 Å, with an indefinite length. These filaments extend in a straight line across the anisotropic and isotropic bands and thus across the successive sarcomeres (Fig 137). When the muscle is studied after extraction by the mild alkaline-saline solutions employed for the extraction of the myosin, filaments are obtained which are only visible with the electron microscope and

which present characteristics similar to those found in the myofibrillae.

The fact that these filaments of myosin are continuous through the entire sarcomere and even in adjacent sarcomeres has led to the belief that there exist in the A band other substances which increase the electron density. It has been suggested that this property may depend upon a greater content of potassium (Macallum) and on mineral salts (Scott). Nevertheless, this problem is still in dispute (Engström).

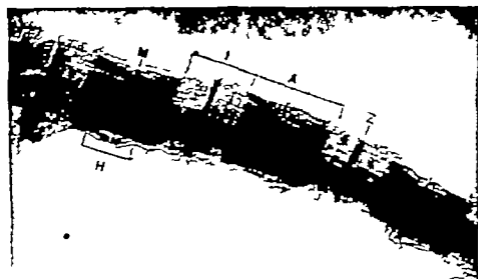


Fig. 137 Electron micrograph of a single myofibrilla from frog sartorius, extended and fixed in formalin. Stained with phospho-tungstic acid and shadow cast with chromium  $\times 15,000$  I isotropic band, A anisotropic band Z, Z line M M line H Hensen's disc. (Courtesy of M. A. Jakus and C. E. Hall and of J. Biol. Chem. 167: 705-194.)

At present the relative isotropy of the I band remains unexplained. Since the submicroscopic filaments similar to those of the A band course continuously through it, it has been suggested recently that this relative isotropy may be due to the presence of a lipid component which would neutralize the positive anisotropy of the filaments (Dempsey et al.) or perhaps to a nucleic acid component (Banga).

In recent years notable advance has been made in the study of the proteins of muscle. This progress has had a profound effect on our concepts of the physiology of muscular contraction and on the submicroscopic study of the myofibrillae (see Szent-Györgyi). These investigations have shown that myosin, formerly considered to be a single protein which constitutes 50 to 70 per cent of the total protein of the muscle, is in reality a complex of two proteins which have different properties. Myosin (in the old sense) has been called *actomyosin* and its two components *actin* (Straub).

and *myosin*. Actin has the peculiar property of undergoing a reversible change from the globular to the fibrous state and vice versa, depending on the pH and the ionic strength of the medium. In Chapter IV we discussed the importance of the

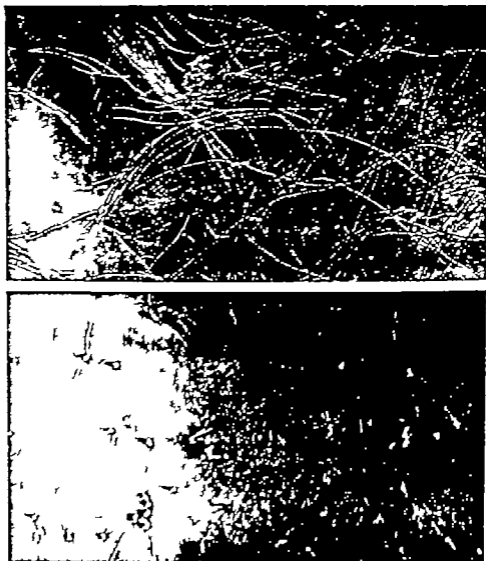


Fig. 138. Electron micrographs of actin extracted from rabbit muscle. *Upper* pH about 5.7 Shows long filaments 80 to 100 Å in width, *Lower* pH between 4.5 and 5.0 Filaments are fragmented into short rods. Shadow-cast with chromium.  $\times 19,000$ . (Courtesy of M. A. Jakus and C. E. Hall and of J Biol. Chem. 167 705, 1947)

globular fibrous (G F) transformation in biological phenomena. In the case of actin, the electron microscope, confirming the previous results obtained by studies of viscosity and flow birefringence, demonstrated that this G F transformation is due to the reversible aggregation of particles into elongated filaments. At pH 6.5 the filaments are many micra in length and 80 to 140 Å in width at pH 5.7 the length is diminished (Fig 138) but

the width remains constant. At a lower pH the filaments are broken down into short rods. Myosin (in the new sense) is composed of filaments 0.5 to 1.0  $\mu$  long and of uneven contour. When fibrillar (F) actin and myosin are mixed together the actomyosin complex is formed and there is a sudden increase in viscosity. Electron micrographs show long filaments which are wider than the actin ones. This seems consistent with the idea by Szent-Györgyi, that myosin adheres to actin filaments to form the actomyosin complex (Jakus and Hall). Although we cannot discuss here the importance of these discoveries in the light of recent

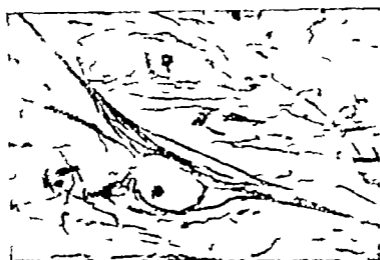


Fig. 139 Nerve cells showing neurofibrillae. Silver impregnation.

studies on the physiology and enzyme chemistry of muscular contraction (see Ferni, Szent-Györgyi) it can be said that in the submicroscopic structure of the myofibrillae we have one of the best examples of the coupling of energetic processes with the actual machinery involved in function (muscular contraction). In this case, form and function are so intimately related in the realm of molecular organization that one cannot be separated from the other (see Introduction).

Nerve cells differentiate very early in embryonic development and show extensions of the cytoplasm—the dendrites and the axon—the latter continuing into the nerve fiber. In fixed and stained preparations fine filaments called *neurofibrillae* can be demonstrated in the cytoplasm of the cell running in all directions and continuing into the dendrites, axon and nerve fiber (Fig. 139). Although fibrillae have been observed in living ganglion cells cultured *in vitro* (Weiss and Wang) they are generally invisible in living cells even when darkfield illumination is used.

This fact has given rise to a controversy concerning the true significance of neurofibrillae and has also led some to think that they are artifacts due to fixation. This uncertainty regarding the microscopic organization of the nerve cell and fibers is reflected in the theories referring to conduction of the nerve impulses. Neurofibrillae have been considered as merely supporting or trophic structures with no role in nerve conduction (Parker) and the polarized membrane supposed to be involved in the process of conduction has been located by nerve physiologists at the periphery of the nerve axon and even at the Ranvier nodes of the nerve fibers (von Murali).

The meager and uncertain results obtained by histological and cytological methods make advisable the use of submicroscopic techniques of structural analysis. The structure of the living axoplasm of giant nerve fiber of the squid was analyzed by the use of polarization optics, and a weak positive birefringence along the axis of the nerve axon was found (Bear, Schmitt and Young). These results indicated the presence of submicroscopic fibrillae oriented parallel to the axis.

Very recently an electron microscope study of the submicroscopic organization of the nerve axon has shown that a variety of vertebrate and invertebrate myelinated and unmyelinated nerve axons contain a characteristic fundamental structure which apparently consists of tubular elements of indefinite length running parallel to each other and to the axis of the axon. These elements have been named *neurotubules* (De Robertis and Schmitt).

The diameters of the neurotubules in the nerves of marine invertebrates such as the lobster and *Lamulus* lie between 200 and 300 Å. In the giant fiber of the squid, neurotubules of about 300 to 400 Å are found. In frog motor roots, in the human sympathetic and in the sciatic nerve of the rabbit, wider neurotubules are observed.

In human sympathetic fibers neurotubules ranging between 400 Å and 1100 Å are found with a mean width of about 640 Å. Neurotubules show a core of low electron density and a wall with a higher electron density. This differential is accentuated by an electron stain such as phosphotungstic acid. Although in some cases the core of the tubules appears to be continuous (Fig. 140) it is also possible to see a periodic structure. This consists of thin transverse bands crossing at regular intervals from one side to the other (Fig. 141). In human sympathetic nerves the main period is about 650 Å with a range from 450 Å to 750 Å. Between the main periodic bands at least two fainter lines can be

observed. The periodic axial unit consists then of three bands which have been called  $\alpha$ ,  $\beta$  and  $\gamma$ . The relative density, width and position within the period depends not only on the type of nerve from which tubules were obtained but also on conditions of preparation and staining. In some cases, under special technical



Fig. 140. Electron micrograph of neurotubules from the axon of human sympathetic fibers. Double contoured tubular elements are seen. Stained with phosphotungstic acid.  $\times 35,000$ . (De Robertis and Schmitt.)

conditions, it is possible to see a larger number of bands. In figure 141 a total of seven bands may be seen. Bands are also seen after shadow casting as transverse elevations on the surface (Fig. 141). In human sympathetic nerves neurotubules are packed together parallel to each other, leaving only narrow interstices. The individual tubules arrange themselves so that the periodic bands of each tubule are adjacent to and in phase with those of their neighbors.

Isolated fixed neurotubules often appear to be surrounded by an amorphous cloud of high electron density which appears to be capable of diffusion away from the wall of the tubule, and which is greatly accentuated by the application of phosphotungstic acid. The material responsible for this peritubular cloud can be washed away readily with distilled water.

Neurotubules are very labile structures rapidly destroyed by distilled water and salt solutions and, in the case of the human

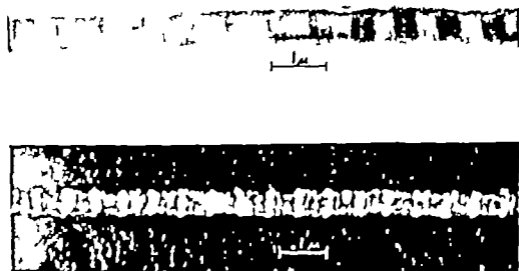


Fig. 141 Electron micrographs of neurotubules of rabbit sciatic nerve fibers. Above: Intensely stained with phosphotungstic acid, 7 bands are seen. Around the neurotubule a small amount of amorphous peritubular material is found.  $\times 114,000$ . Below: Shadowed with chromium, bands appear on the surface of the neurotubule as transverse elevations.  $\times 90,500$ .

sympathetic, they break down after a few hours of standing in a refrigerator. The structure of the tubules can be preserved by the use of formaldehyde fixation. However, neurotubules can be observed without fixation in fresh material dried on the grid.

The periodic and cylindric variations in electron density and in affinity for phosphotungstic acid betoken corresponding chemical differences in various parts of the tubule.

In experiments with bull frog motor roots kept in cold Ringer's solution for varying periods before fixation, it was found that neurotubules appeared normal until the rapid decline of action potential indicated failure of conducting mechanism. Thereafter chiefly disintegration products of the neurotubules were observed. In the wallerian degeneration, which takes place

after the section of a nerve, neurotubules also disintegrate and disappear

The existence of these submicroscopic axonic tubules with their obviously complex chemical constitution in a structure formerly regarded as almost homogeneous may lead to the re-interpretation of physiological data and a recasting of physiological theories relating to the conduction of the nerve impulse

## SENESENCE AND DEATH OF THE CELL

After an initial undifferentiated phase and a phase in which differentiation occurs, almost all cells pass finally into a terminal period of senescence, which ends in death. These last phases of cell life are as yet little known and have not been studied with all of the cytological and cytochemical methods at our disposal. Relatively more is known of cellular pathology which is the study of the abnormal processes which take place in the cell due to the action of various agents or of metabolic, genetic, or other alterations. This study is not, strictly speaking cytology for which reason we shall consider here only those modifications which characterize senescence and death when these processes occur normally without the addition of extraneous factors or perturbations

In studying these problems in cells and tissues we must not confuse them with those of the senility and death of the entire organism, although these are intimately linked to them. Indeed, even in the embryo there are cells which are continually undergoing a senescent process and dying and the entire individual is enveloped by dead cells (external layers of the epidermis) which serve for protection. But on the other hand, the death of the individual does not imply the immediate cessation of the vital phenomena in all of its cells. Thus it is known that the cells of the trachea and of the bronchi continue their ciliary movement for a long time after the heart has ceased to beat and that the leucocytes continue their ameboid activity. In laboratory animals which have been sacrificed it has been found that certain cells survive as long as 120 hours after death (Lewis and McCoy). Furthermore many cells transplanted at the time of the death of the individual can continue to live in other individuals as hosts or in tissue cultures. Nevertheless, it is evident that the death of cells of vital importance to the organism such as those of the nervous tissue finally causes the death of all the remaining cells.

The study of senescence in various cells and tissues demonstrates that those elements which retain the capacity of dividing

continuously do not grow old. Division and growth of cells apparently involve a physicochemical renovation of the cell and prevent the phenomena of senescence. This is true in the unicellular organisms, which generally reproduce in a continuous form and therefore are potentially immortal. The same thing happens with certain cells cultivated *in vitro*. The famous culture, begun by Carrel, of cells from a fragment of chicken heart continues to grow after thirty-six years! Its cells need only the renewal of the nutritive fluids (see Chapter III) to continue to live an apparently endless existence. On the other hand, very well differentiated cells, such as those of the nervous tissue, generally lose the capacity to reproduce (and therefore the capacity to be cultivated *in vitro*) and are condemned ultimately to senescence and death.

Cowdry has classified cells into four groups on the basis of their degree of differentiation and ability to divide. The first group, *vegetative intermitotic cells* comprises those undifferentiated cells which have the capacity to multiply continually, to give origin to other cells of the same group or to elements which undergo a later differentiation and proceed to form part of the second group. Typical examples are the basal cells of the epidermis, the division of which gives origin to other similar cells or to cells which differentiate to form the elements of the other more superficial layers, the spermatogonia of the testis, which, by division, give rise to other spermatogonia or to cells further advanced in the germinal series (spermatocytes, spermatids), the hemocytoblasts of the bone marrow and so on. In all these cases cell life passes without differentiation between two successive mitoses. In these cells the changes with age are very limited or even absent, because of the fact that the individual life is very short and is not accompanied by any structural differentiation, nor by any diminution of vitality.

In the second group, or *differentiated intermitotic cells* the cell between two mitoses is undergoing a progressive differentiation which generally terminates by its transformation into an element of the third or fourth type. In the case of the testis, between the spermatogonia (vegetative intermitotic cell) and the spermatozoon (fixed postmitotic cell) there is a series of generations of differentiated intermitotic cells including the spermatocytes and spermatids. In the same way in the bone marrow there is a series of intermitotic generations which are progressively differentiating to constitute the terminal elements, erythrocytes and leucocytes.

Among these cells, as in the former group, the individual

life continues between two mitoses, and, although differentiation progresses in them continuously the changes due to age are small or lacking

The two last groups comprise differentiated, terminal, or post mitotic cells, that is, elements which generally die without further division. Within this category two different degrees may be distinguished reversible postmitotic cells and fixed postmitotic cells

The *reversible postmitotic cells* are those such as the hepatic, renal and thyroid, which do not normally divide, but which, in cases of extraordinary necessity for example, after the extirpation of a large part of the organ, will do so. Among these cells, there may be observed various manifestations of senility

The *fixed postmitotic cells* reach the highest degree of specialization and undergo senescence and die without being able to divide anew. Such is the case with the nerve cells, the rod and cone cells of the retina the erythrocytes, and so forth. Among these cells, some (erythrocytes, leucocytes, and so on) have a relatively short life and, for this reason, there are differentiated intermitotic cells which are continually replacing them. On the contrary others (nerve cells and the like) have a very long life and, for this reason, very early lose the capacity to reproduce (at the end of the first year in the case of human nerve cells). It is in such cells that the changes due to age are most evident.

When a cell undergoes senescence, a series of modifications is produced in it which have been grouped under the common term *cytomorphosis* (Minot). One of the most characteristic changes of senility is the accumulation of the pigment of exhaustion (sometimes also called wear and tear pigment or *abnutzungs pigment*) which is observed particularly in the nerve cell and in the myocardial tissue and in lesser degree in the cells of the liver kidney testis, ovary thyroid, and so on (Chapter VI). Recently the value of pigmentation as a constant sign of the aging of nerve cells has been a subject of discussion. It is believed, in general, that the accumulation of pigment in senile cells is due to the progressive difficulty which the cells have in excreting poorly soluble products and that this functional alteration is an important factor in cytomorphosis. Other common cytological changes are the accumulation of small droplets of fat, the diminution of the chromidial substance (in the nerve cells) and the diminution of cell volume (hypotrophy). This last change is very considerable in striated muscle fibers, but it is difficult in this case to decide whether it is due exclusively to senescence or also to the lack of activity.

Tissue culture techniques permit the study of cytomorphosis in a very favorable way because aging processes and death may



Fig 142 Life cycle of myeloplax (polykaryocyte) in a benign myeloplaxoma. Above young cell. Center- adult cell. Below senile phase. Pyknotic nuclei with their irregular outlines are seen. The cytoplasm has become darkened and retracted. (Courtesy of D. Brachetto-Brian)

occur in the course of a single cultivation. Cultured cells, like the whole organism, pass through a series of stages but at a much faster rate. The stages might be listed as follows (1) 'increase in

mass, (2) differentiation and organization, (3) equilibrium, (4) senility and (5) death and dissolution. Aging starts by a remission of growth and adjustment to a minimum activity, the formation of intercellular substance, increase in the death quota of the cells, reduction in residual growth energy and finally by morphological cell changes (Fisher). These changes are first seen at the periphery of the culture and are generally characterized by the vacuolization and infiltration of the cytoplasm with fat and are followed by cell disintegration.

From the point of view of the duration of life and of the rapidity with which cytomorphosis occurs, the cells and tissues have been divided into *labile* and *stable* or *perennial* categories (Bizzozero). In the labile tissues the cell renovation is rapid and their elements are continually undergoing senescence, death and replacement. The stable tissues, such as muscular and nervous tissues, are not generally renewed and their cytomorphosis, which is very gradual, presents the changes described above.

In the labile tissues the regressive steps are accelerated and follow various paths. Thus, in simple epithelia the protoplasm becomes less viscous and is liquefied, whereas, in stratified epithelia there is an increase in viscosity and gelation. In the cells of the sebaceous glands an infiltration and fatty degeneration takes place in the cytoplasm and every cell disintegrates and is converted into sebaceous matter.

In cytomorphosis the nucleus often stains more intensely and is shrunken simultaneously its structural details are progressively lost. This process which is called *nuclear pyknosis* (Gr. *Pyknos* dense) leads to the death of the cell (Fig. 142).

The senescence of the cell has been studied with great detail, particularly in the nerve cells. In normal senility these elements show constantly a retraction of the cell boundaries, a change in the nucleus, consisting in the loss of transparency of the nuclear sap, a notable diminution of the chromidial substance of Nissl (Fig. 143) and a fragmentation of the Golgi apparatus (golgiolysis). Furthermore, in some species a tendency is seen for the nucleus to divide by amitotic division (for example, division of the nucleolus, lobulation of the nucleus and even a true division) (Andrew). The process of senescence of the epidermal cells has been studied recently with numerous histological and cytochemical techniques. The changes in the viscosity of the cell protoplasm have been studied also by means of ultracentrifugation (Cowdry).

Recent studies tend to show that the histological and cytological picture of old age in the individual organs is not the result

of "simple processes of degeneration and atrophy. The changes occurring seem to be relatively complex and to depend, not only on atrophy of cells, but on interactions among the different cell types under the conditions of senescence of the organism.

Thus, in the nervous system, while the nerve cells show a series of degenerative changes and many of them actually die and disappear, some of the neuroglial cells (particularly the satellite oligodendroglia and microglia) actually increase in num



Fig. 143 *Left* Large motor cell from the ventral horn of the spinal cord of a young mouse. The Nissl material is abundant. The nucleolus stains deeply. *Right* Large motor cell from the ventral horn of a senile mouse. The Nissl material is scanty. The nucleolus is lightly stained. A rather coarse vacuolation of the pale cytoplasm is apparent. (Courtesy of W. Andrew)

bers and appear to participate in the removal of dead nerve cells. Furthermore, a marked proliferation of the relatively undifferentiated cells of the intralobular ducts has been seen in the pancreas. This process leads to the formation of numerous cyst-like spaces by the multiplying duct cells (Andrew).

While fibrosis is a conspicuous feature of senility in some organs, as in the thyroid gland, it is almost nonexistent in others, as in the liver. In the latter organ giant nuclei and intranuclear inclusions are often seen.

The varied and complicated senile changes occurring in the different organs make necessary a detailed histological and cytological study of the process in each type of tissue.

## Theories of Cell Senescence

We are as yet very far from knowing the causes which determine the aging of the cell. Various theories have been elaborated which, in general, deal only with partial aspects of the problem. Some of these stress the role of *endogenous factors* by which we mean the progressive and irreversible alterations of the protoplasm. Others give greater importance to *exogenous factors* or modifications of the surrounding medium. Finally, a third group affirms that the cell has in itself the factors which limit the duration of its life.

Among the theories of the first group we may mention that which suggests that senescence is due to an alteration of the *colloidal state* of the protoplasm (Ruzicka and collaborators). In Chapter II it was said that many colloidal solutions undergo a phenomenon of *syneresis* due to the action of time or of other factors. According to this theory, similar phenomena would be produced in the protoplasmic colloid which lead to a diminution in the degree of dispersion with loss of water and of electrical charge. This process as a whole is called *protoplasmic hysteresis*. Other conspicuous colloidal modifications which take place particularly during embryonic development are the increase of protein substances which, like keratin and plastin, are resistant to digestion and dissolution by enzymes, the decrease in water content, and increase in consistency of the cell.

The process of cornification, which occurs in certain stratified epithelia, can be considered as a rapid phenomenon of cytomorphosis. This starts at the basal cells which divide by mitosis and continues toward the surface where cornification and death ensue. Simultaneously the protein content of the cells increases and the isoelectric point of the proteins is displaced from the acid side toward the neutral point (Zeiger).

Other authors give considerable importance to the accumulation of residues and particularly of the pigments of exhaustion which were mentioned above. This accumulation, along with condensation of colloids and diminution of cell permeability, might disturb the total metabolism of the cell. However, one may raise the question whether the accumulation of pigment is the cause of senescence or simply one of its manifestations.

The medium in which the cell lives also seems to have considerable importance, and changes in its composition may influence the development and senescence of the cells. In speaking of the environment of the cell (internal medium) we do not refer to the circulating blood. This is characterized by the presence of

multiple regulatory mechanisms which maintain constant its temperature, sugar content, acid base equilibrium and so forth (homeostasis of Cannon) The great majority of the cells are not bathed directly by the blood but by the interstitial fluids, the composition and properties of which not only are distinct from those of the circulating medium but variable in each particular tissue.\*

With aging, the regulatory mechanisms which maintain the uniformity of the blood are disturbed. At the same time, the various tissue fluids are altered, and the changes may not be the same in every case (Cowdry) Thus the senescence of the cells is accompanied by a senescence of the internal medium, and these two processes not only appear to be correlated but also inter dependent.

In cells cultured in vitro, senility of cells is primarily due to external factors such as accumulation of metabolic products or lack of foodstuffs When those causes are removed by transfer or change of the medium, rejuvenation of the cells occurs However in the plasma of senile individuals there are other factors that may affect the life history of cultured cells

The studies of Carrel and collaborators have shown that the blood plasma not only contains growth promoting substances which stimulate the growth of a tissue culture, but also contains inhibitory substances These latter increase with the age of the animal and reach a maximum concentration in old age. These inhibitory substances could be considered as the cause of cell senescence However it must not be forgotten that tissue fluids are produced by the cells so that these inhibitory substances may be the consequence and not the cause of senescence. Although this problem has not been completely resolved, it is evident that the internal media contain at least one of the factors which limits cell proliferation (See Fisher )

A third group of authors attempts to explain senescence on the basis of factors which lie in the cell itself and determine irreversible vital cycles According to this view, certain cells of the organism not only have a fixed determination of their destiny and specific functional and morphological differentiation but also have a predetermined limit to their life potentiality By the study of tissue cultures it has been possible to demonstrate that various tissues, although cultivated under the most favorable known conditions, do not survive more than a certain period of time (two months in the case of cultures of kidney eighteen months in that

In reality the optimum internal medium varies for each type of cell. Thus the tissue cultures have demonstrated that the macrophages multiply actively in plasma, while fibroblasts and epithelial cells do so with considerable difficulty

of epithelium of the iris, a few weeks for the pancreas, and so on). In these cases the vital capacity of the cells in such a medium seems to be strictly limited. All these various types of tissues are found in the organism, including those which, when grown in favorable culture media, divide continually and do not age and those which can be cultivated only for a short time or which do not usually reproduce (nervous tissue). The causes which limit the life of the cells are unknown but they seem intimately related to the differentiation and functional specificity of the cells, and possibly to hereditary factors (lethal genes). It seems possible that the life span of different types of cells might be determined in part by heredity.

As we said at the beginning it is too early to make general interpretation of the causes of cell senescence since there is lacking a detailed analysis of the process in the various tissues. It is possible that, besides the internal conditions which limit the duration of the life of the cell, humoral factors may play a role and that the overload of residues and the protoplasmic hysteresis may accelerate the process of senescence (Ries).

## Cell Death

The senescence of the cell leads finally to catabiosis (Gr *kata*, down *Bios* life) and to death. Here also the histopathological literature is copious and, in contrast, precise definitions and a clear knowledge of the purely cytophysiological alterations are lacking. In dealing with the death of the cell it is at times difficult to separate the strictly physiological from the pathological processes.

Cell death is generally defined as the irreversible cessation of the vital phenomena. Nevertheless the determination of the instant at which a cell ceases to live is at times very difficult. Except when death is produced rapidly by agents which cause a speedy coagulation or precipitation of the protoplasm (as in the case of fixation, or of death by heat, or by various toxic agents) the cessation of the cellular processes occurs in a gradual way. A cell may undergo an irreparable injury and nevertheless, some of its functions may persist for a certain length of time. If a tissue of any type is ground until all of the cell boundaries are destroyed (bri) several of the metabolic phenomena such as the consumption of oxygen, fermentation and glucolysis may persist. Nevertheless when these phenomena are studied in relation to time appreciable differences are noticed. In the bri the majority of metabolic processes decrease with relative rapidity and dis-

appear while in the intact cell these are maintained at the same level for as long as cell vitality is conserved. (See Chapter X.)

Cytologically, a retraction of the cytoplasm or a characteristic alteration of the nucleus may often be noted, but these changes are found only when the process is very far advanced

In some cells the lack of reproduction, growth, or movement may be important criteria of death. However, they do not have a general value because in many cells and tissues these processes are not observed even in the living state. Variations in the oxidation reduction potential or pH also have no value, although in dying cells there is generally an acidosis. With the ultramicroscope it is often possible to see the appearance of brownian movement. However, this phenomenon may be apparent in living cells. Dead cells seem to absorb ultraviolet light more intensely.

A cytological criterion of cell death which is usually considered reliable is the diffuse staining of the cytoplasm and of the nucleus by vital dyes (neutral red, methylene blue, and so forth). While in a living cell these stains are accumulated in circumscribed granules or vacuoles in the cytoplasm, after death the cytoplasm and the nucleus stain intensely and in a diffuse manner.

The following criteria have been considered as identifying cell death by means of supravital staining with neutral red: (1) loss of color from the granules and vacuoles, (2) diffuse pink staining of the cytoplasm and nucleus, (3) the appearance of a sharp and distinct nuclear membrane and a change in the texture of the cytoplasm and nucleus (Lewis and McCoy).

In cultured cells the following changes are ordinarily considered as signs of death: (1) Retraction of pseudopods and rounding up of the cell, (2) coagulation and shriveling of the nucleus, (3) dissolution of the chondriosomes and (4) diffuse vital staining of the cytoplasm and nucleus (Levi).

### Postmortem Modification of the Cell

When death of the cell occurs in a rapid manner and all enzyme activity ceases at the same time (fixation, death by heat, and so on) the cell structure does not undergo postmortem alterations. Such a happening is, however, exceptional and, in general, postmortem phenomena of an autolytic character do occur after death.

In the study of the modifications which occur during the death of the cell we must distinguish those which precede and lead to such a death from those which occur postmortem. The

former are grouped generally under the name *necrobiosis* (Gr *Nekros* death *Bios* life) when death is slow and gradual

Some of the necrobiotic changes were described along with the discussion of senescence of the cell. It was said at that time that these vary from one type of cell to another. In certain cases the viscosity of the cytoplasm is diminished, as evidenced by the appearance of brownian movement (sol). Finally liquefaction occurs (*necrobiosis by liquefaction*). In others the viscosity is increased and the protoplasm gels (*gelation*) or is coagulated (*necrobiosis by coagulation*).

In many cells, death is preceded by a shorter or longer period characterized by an alteration of the normal metabolism and by degenerative changes of fatty waxy, hydropic, pigmentary or other types of degeneration. Since these processes are more frequently due to external factors (toxic, infectious and the like) their study in detail belongs to the realm of general pathology.

The *postmortem modifications* generally result from the activity of intracellular enzymes which begin their action after death. These enzymes are of a hydrolytic nature and attack and break down the large molecules of the cell particularly protein molecules (proteolysis). At the same time the lack of oxygen favors anaerobic fermentation and the formation of various acids, especially of lactic acid. The accumulation of small molecules and ions resulting from autolysis increases the osmotic pressure and causes the entrance of water with resulting swelling of the cell.

It is generally admitted that one of the phenomena which follow death is the irreversible coagulation of the protoplasm. This may persist for a long time, but commonly it is followed by the digestion and liquefaction of the cell.

Frequently liquefaction is preceded by the swelling of the cell and by the appearance of protein granules in the midst of the cytoplasm, which give to it a turbid and characteristically dusty aspect (*cloudy swelling*). This process may occur also in living cells which have been altered by the influence of toxins, infectious diseases, and so on. In this case we are dealing with a degenerative change which may be reversible.

The cloudy swelling of the cell is preceded by the accumulation of acids in the protoplasm and by the lowering of the pH (acidosis). This accumulation of ions increases the imbibition of water and at the same time brings about precipitation of the proteins of the cytoplasmic matrix in the form of fine granules. It is also common to find the appearance of minute droplets of fat, which are produced by the dissociation of the lipoprotein complexes (*lipophanerosis*).

The cloudy swelling may be reversed when it is due to degeneration. When the alteration is of a postmortem character, it is generally followed by a progressive imbibition of water and finally by the disintegration and dissolution of the cell.

Cloudy swelling should not be confused with a similar appearance of the cytoplasm which may be produced by the alteration of the chondriome. We know (Chapter V) that this organoid is one of the most sensitive parts of the cell and that after death it rapidly fragments into granules which undergo swelling. In this case the granular appearance of the cytoplasm is brought about by the change in the chondriome. In cloudy swelling the granules result from the separation of the phases and the precipitation of the proteins of the ground cytoplasm.

In postmortem alterations the *behavior of the nucleus* differs from that of the cytoplasm. In general, its structure and stainability resist the autolytic phenomena for a longer time and, in many cases, the stainability may even increase, possibly because of the separation of the nucleic acid from the protein which results in the liberation of acidic groups (see Chapter VII). This process called *pyknosis* is generally accompanied by a shrinkage of the nucleus and the disappearance of its structural details (perhaps due to the dissolution of the nucleic acid into the nuclear sap).

Nucleases come into action later and break down the nucleic acid molecules. The nucleus then loses its stainability and dissolves (*karyolysis*) either with or without previous nuclear fragmentation (*karyorrhexis*).

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